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Cornelius *et al.* Examiner: Mitchell, G.W.

Application Serial No.: 10/605,406 Publication No.: 2005/0070601

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Title: Psychostimulant Effects of Forskolin Including Anorexia

May 31, 2005

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THIRD-PARTY SUBMISSION IN PUBLISHED APPLICATION PURSUANT TO 37 C.F.R. §1.99

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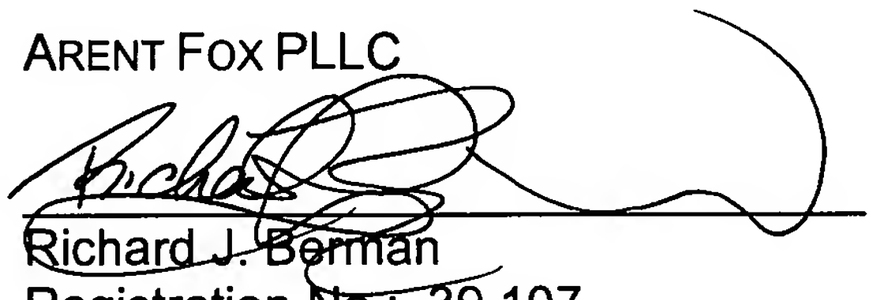

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LIST OF REFERENCES CITED BY THIRD PARTY

(Use several sheets if necessary)

U.S. PATENT DOCUMENTS

EXAMINER'S INITIALS		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE
	AA						
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OTHER REFERENCES *(Including Author, Title, Date, Pertinent Pages, Etc.)*

	AE	Choquet *et al.*, "Is forskolin a stimulant of gastric secretion?" *C R Seances Soc Biol Fil.* **182(3)**, 335–343, 1988. (article in French)
	AF	English language abstract of AF.
	AG	Lambert *et al.* "CART peptides in the central control of feeding and interactions with neuropeptide Y", *Synapse* **29(4)**, 293–298, 1998.
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	AJ	Kreider, "Coleus forskohlii fat loss update", *Muscular Dev.* **39(2)**, 260–262, 2002.
	AK	Kreider *et al.*, "Effects of Coleus Forskohlii Supplementation on Body Compostion and Markers of Health in Sedentary Overweight Females", *FASEB J.* **16 Suppl: 59** (LB305), 2002. (abstract only)
	AL	Edwards *et al.*, "BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis", *J Lipid Res.* **43(1)**, 2-12, 2002.
	AM	Dominguez *et al.*, "Characterization of the cocaine- and amphetamine-regulated transcript (CART) peptide gene promoter and its activation by a cyclic AMP-dependent signaling pathway in GH3 cells", *J Neurochem.* **80(5)**:885–893, 2002.
	AN	ForsLean® Food Intake Reduction Study; available at http://www.forslean.com/food.htm; study conducted between April 3, 2003 and May 16, 2003.

EXAMINER	DATE CONSIDERED

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AE

Société de Biologie de Montpellier. **Séance du 12 novembre 1987**

C. R. Soc. Biol., 1988, *182*, 335-343.

Pharmacodynamie.

La forskoline est-elle un stimulant de la sécrétion gastrique ?

par ARMELLE CHOQUET, RICHARD MAGOUS, JEAN-CLAUDE GALLEYRAND et JEAN-PIERRE BALI

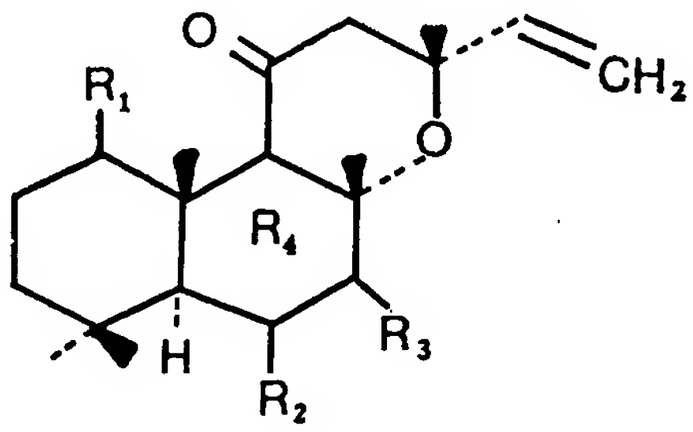
Laboratoire de Biochimie des Membranes du CNRS UPR-41, INSERM U-249, Faculté de Pharmacie, 15, avenue Charles Flahault, 34060 Montpellier Cedex

(reçue le 6 juin 1988).

Summary. — The diterpene, forskolin, direct activator of the catalytic subunit of the adenylate cyclase from various tissues, also stimulates gastric acid secretion: *in vitro*, with an isolated parietal cell preparation, forskolin dose-dependently stimulated acid secretion (EC_{50} : 1 μM) (measured by accumulation in the acidic spaces of the weak base [^{14}C]-aminopyrine) and the maximal acid secretory value at 0.1 mM was 4 times higher than that obtained with histamine. Forskolin drammatically increased the production of intracellular cyclic-AMP at a level 4 times higher than that obtained with histamine at the same concentration. *In vivo*, gastric acid secretion of the rat is dose-dependently increased. The doses required to get a significant response (100 nmol/kg) were 1 000 times higher than those required for gastrin and 100 times lower than those for histamine, but the same maximal value was obtained. Cimetidine did not significantly modified this response. These results demonstrate that, both *in vitro* and *in vivo*, forskolin is a potent stimulant for gastric acid secretion.

Résumé. — La forskoline, diterpène activateur direct de la sous-unité catalytique de l'adénylcyclase membranaire de nombreux tissus de Mammifères, est capable de stimuler la sécrétion gastrique acide : *in vitro*, sur une préparation de cellules pariétales isolées, la forskoline stimule de façon dose-dépendante la sécrétion acide (EC_{50} : 1 μM) (mesurée par accumulation de la base faible [^{14}C]-aminopyrine dans les espaces acides) mais le maximum de sécrétion obtenu (pour la dose de 0,1 mM) est environ 4 fois supérieur à celui obtenu pour l'histamine. Parallèlement, la forskoline stimule la production d'AMP cyclique intracellulaire à un niveau 4 fois supérieur à celui obtenu avec la même dose d'histamine. *In vivo*, la sécrétion acide gastrique est augmentée de façon dose-dépendante. Les doses de forskoline nécessaires pour obtenir une réponse (100 nmol/kg) sont 1 000 fois supérieures à celles de gastrine et 100 fois inférieures à celles d'histamine; le maximum de sécrétion obtenu est le même. La cimétidine ne modifie pas cette réponse de façon significative. Ces résultats démontrent que la forskoline, aussi bien *in vitro* qu'*in vivo*, se comporte comme un puissant stimulant de la sécrétion gastrique.

La forskoline est une drogue d'origine végétale extraite des racines d'une plante indienne *Coleus forskohlii.* Elle est constituée de cinq isomères (Fig. 1); les isomères A et C sont les constituants majeurs



	R_1	R_2	R_3	R_4
A	H_2	◀OH	◀OAc	H
B	H_2		◀OH	H
C	•••OH	◀OH	◀OAc	OH
D	•••OH		◀OH	OH
E	•••OH	◀OAc	◀OH	OH

FIG. 1. — Structure des 5 diterpènes extraits de *Coleus forskohlii.*
Les isomères A et C sont les constituants majeurs de l'extrait. C, D et E possèdent des propriétés cardiotoniques intéressantes.

de l'extrait et seuls les isomères C, D et E présentent une activité pharmacologique sur le système cardiovasculaire (inotrope positif, hypotenseur, inhibiteur de l'agrégation plaquettaire), sur les sécrétions hydroélectrolytiques (salivaire, colique), sur la stéroïdogenèse et sur la lipolyse (1, 2, 3). *In vitro*, le diterpène C se présente comme un stimulant puissant de la sous-unité catalytique de l'adénylcyclase membranaire (4) avec une EC_{50} de 10 μM. Ce composé augmente la formation d'AMP cyclique (Tableau I) dans le cerveau, les glandes salivaires, les glandes gastriques, les acinis pancréatiques, les adipocytes, les plaquettes sanguines et les cellules surrénaliennes. La stimulation observée est d'intensité variable suivant le type de tissu : dans l'estomac, elle est faible et de moindre importance que celle obtenue par les ions F^-. Cette activation est directe, sans intervention d'une G-protéine (5).

TABLEAU I. — Effets de la forskoline et du NaF sur l'activité adénylcyclase de divers tissus.

Tissu	AMP cyclique en pmol.min^{-1}.mg^{-1}		
	Basal	NaF	Forskoline
Cortex	65 ± 6	450 ± 8	630 ± 20
Cervelet	110 ± 2	260 ± 16	810 ± 4
Striatum	130 ± 8	230 ± 12	1 970 ± 90
Cœur	10 ± 0,6	41 ± 3	100 ± 5
Foie	11 ± 0,4	54 ± 12	150 ± 4
Muscle squelettique	9 ± 2	38 ± 3	19 ± 0,2
Surrénales	24 ± 0,8	200 ± 9	81 ± 2
Pancréas	14 ± 0,5	120 ± 2	33 ± 0,6
Grêle	20 ± 1	68 ± 1	44 ± 11
Rate	37 ± 3	190 ± 20	160 ± 6
Reins	22 ± 2	104 ± 10	98 ± 3
Estomac	13 ± 1	85 ± 5	23 ± 1
Testicules	15 ± 1	60 ± 2	37 ± 2
Poumons	40 ± 3	310 ± 30	64 ± 3

Au niveau des glandes gastriques isolées, certains travaux réalisés *in vitro* (6, 7) avaient montré que la forskoline était capable d'activer la sécrétion d'acide et de pepsinogène avec une augmentation concomitante des niveaux d'AMP cyclique et de l'activité des protéines kinases dépendantes de l'AMP cyclique. De plus, ces mêmes auteurs avaient montré que la sécrétion acide et la respiration cellulaire stimulées par la forskoline étaient inhibées par la cimétidine, suggérant qu'elle agissait également en potentialisant l'action de l'histamine endogène. Cependant, aucune expérimentation *in vivo* n'est venue compléter ces observations.

Nous nous sommes proposé dans cette étude d'examiner en parallèle l'effet de la forskoline sur la sécrétion acide du rat anesthésié à estomac perfusé selon Ghosh et Schild (8) et sur la captation de la [^{14}C]-aminopyrine et les niveaux d'AMP cyclique intracellulaire par les cellules pariétales gastriques isolées de la muqueuse fundique de Lapin (9) de manière à préciser l'effet propre de la drogue sur la sécrétion d'acide gastrique.

Matériels et méthodes. — La forskoline (7β-acétoxy-8,13-époxy-1a, 6β, 9-trihydroxylabd-14-en-11-one) provient de Sigma, saint-Louis (USA) ainsi que l'histamine et l'IBMX (3-isobutylméthylxanthine). La gastrine (Nle^{15}-HG-17) est un don du Professeur Moroder du Max-Planck-Institut de Munich. La solution saline de Earle provient de Biomerieux (France) et la collagénase (0,8 U/mg) de Serva (Heidelberg, RFA). La diméthylamine-[^{14}C]-aminopyrine (118 mCi/mmol) provient d'Amersham (Grande-Bretagne).

A. PRÉPARATION DES CELLULES ISOLÉES. TEST A LA [^{14}C]-AMINOPYRINE. NIVEAUX INTRACELLULAIRES D'AMP-CYCLIQUE. — Les cellules de la muqueuse fundique de Lapin ont été isolées par digestion enzymatique avec la collagénase et l'EDTA selon une méthode déjà décrite (9). En bref, la muqueuse est grattée et les fragments de tissus finement hachés sont dispersés dans un milieu A (132 mM NaCl, 5,4 mM KCl, 5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1,2 mM $MgSO_4$, 1 mM $CaCl_2$, 25 mM HEPES, 0,2 % glucose, 0,2 % BSA, 0,02 % rouge de phénol, ajusté à pH 7,4) contenant 0,25 mg/ml de collagénase et 0,3 mg/ml de pronase. Après 15 min d'incubation à 37° C, le milieu est enlevé, les fragments de tissu sont lavés dans un milieu A sans $CaCl_2$ ni $MgSO_4$ et contenant 2 mM EDTA, puis incubés 10 min à 37° C. Les fragments sont ensuite incubés à nouveau avec 0,25 mg/ml de collagénase dans du milieu A pendant 15 min à 37° C suivie d'une autre incubation de 20 min dans le même milieu à la même température. Toutes ces opérations sont réalisées sous atmosphère d'un mélange d'O_2 et de CO_2 (95 % /5 %). Les cellules obtenues sont centrifugées à 200 × *g* pendant 5 min et reprises dans du milieu B (solution saline de Earle contenant 10 mM HEPES et 0,2 % BSA, ajustée à pH 7,4) et filtrées sur tamis de nylon. La préparation ainsi obtenue contient 40-45 % de cellules pariétales dont 95 % présentent les caractères histochimiques de viabilité. Cette population encore hétérogène est ensuite triée par élutriation centrifuge dans un rotor Beckman JE6-B : trois fractions sont collectées à 2 100 rpm en augmentant le flux de liquide de 24 ml/min (fraction I) à 44 ml/min (fraction II) puis à 68 ml/min (fraction III). Estimées par colorations spécifiques, la fraction I contient plus de 70 % de cellules à mucus (diamètre 9-12 μm), la fraction II (diamètre 12-16 μm) contient 50 % de cellules principales et 50 % de cellules à mucus et pariétales, la fraction III (diamètre 16-20 μm) contient 75 % de cellules pariétales et quelques agglomérats de cellules à mucus.

Pour la mesure de l'accumulation de [^{14}C]-aminopyrine, les cellules (1,5 million par ml) sont incubées dans le milieu de Earle avec 0,05 μCi de [^{14}C]-aminopyrine en présence ou non des divers stimulants pendant 20 min à 37° C sous atmosphère de O_2/CO_2. Des fractions aliquotes (3 fois 0,4 ml) pour chaque point sont prélevées du milieu d'incubation et centrifugées sur 0,9 ml de milieu B glacé à 9 000 × *g* durant 1 min. Le surnageant est aspiré et le culot cellulaire repris par 0,1 ml de $HClO_4$ à 10 %. La radioactivité associée aux cellules est mesurée par scintillation liquide (Kontron Betamatic II). L'accumulation d'aminopyrine est exprimée en pourcentage de la radioactivité totale présente dans l'échantillon.

Les niveaux d'AMP cyclique intracellulaire sont évalués de la manière suivante : les cellules (1,25 million par ml) sont préincubées 5 min à 30° C dans le milieu B. Puis, des aliquotes de 0,4 ml de la suspension sont incubés, avec différentes concentrations de forskoline en présence de 10 μM d'IBMX, pendant 5 min à 37° C. La réaction est arrêtée par addition de 0,1 ml d'acide trichloracétique à 40 % et avec 4 000 cpm/

tube de [^{3}H]-AMP cyclique, Les tubes sont centrifugés pendant 15 min à 4° C à 2 500 × *g*. Les surnageants sont collectés et l'AMP cyclique est extrait par 4 fois 5 ml d'éther saturé d'eau. La phase éthérée est évaporée et le résidu redissous dans 0,5 ml de tampon acétate 0,06 M ajusté à pH 6,2. La radioactivité en [^{3}H] d'aliquote de 0,05 ml de chaque tube est mesurée par scintillation liquide dans ACS (Amersham), pour déterminer le rendement d'extraction. Les quantités d'AMP cyclique sont évaluées par dosage radioimmunologique (trousse de New England Nuclear) et exprimées, après correction du rendement, en picomoles produites par million de cellules pour 5 min d'incubation.

B. MESURE DE LA SÉCRÉTION GASTRIQUE ACIDE *in vivo*. — Les propriétés sécrétoires de la forskoline ont été déterminées sur le modèle du Rat anesthésié à estomac perfusé selon le protocole expérimental de Ghosh et Schild que nous avons modifié (8). Des rats mâles de 300 g anesthésiés à l'uréthane par voie i. p. ont été trachéotomisés. Une première canule est placée dans l'œsophage et une seconde dans le duodénum. Une pompe péristaltique d'un débit de 3 ml/min permet d'établir un circuit fermé gastro-duodénal. Le liquide qui circule dans la cavité gastrique est un soluté propionique-succinique (acide propionique 1,3 mM, acide succinique 1,65 mM et NaOH 3,3 mM, ajusté à pH 5,5) maintenu à 30° C.

La secrétion acide est suivie par enregistrement continu du pH du soluté. Les sécrétagogues sont injectés par voie i.v. et la réponse sécrétoire est enregistrée sur 40 min. La différence entre le pH minimal mesuré et la valeur du pH extrapolée à partir de la ligne de base au même moment est déterminée sur le tracé. Les résultats sont exprimés en µmoles de H^+ sécrétés.

Résultats. — SÉCRÉTION ACIDE DES CELLULES ISOLÉES. — La base faible radiomarquée [^{14}C]-aminopyrine s'accumule dans les espaces acides de la cellule pariétale lorsque le pH du milieu est inférieur à son pKa. Cette accumulation est d'autant plus importante que le milieu est plus acide : cette méthode est un test *in vitro* de la sécrétion acide des cellules pariétales gastriques isolées.

L'histamine augmente de façon dose-dépendante l'accumulation de [^{14}C]-aminopyrine dans les cellules pariétales isolées (Fig. 2). La CE_{50} évaluée graphiquement sur la figure 2 est de 0,5 µM pour la forskoline et de 2 µM pour l'histamine. La forskoline induit une accumulation 4 fois plus élevée d'aminopyrine que l'histamine avec une réponse maximale pour la concentration de 10 µM (10 fois inférieure à celle de l'histamine). De la même manière, le dibutyryl AMP cyclique induit une accumulation d'aminopyrine dose-dépendante avec une CE_{50} de 0,5 mM, soit 1 000 fois plus élevée que celle de la forskoline.

Le mécanisme de stimulation de la sécrétion d'acide mis en jeu est lié à la production d'AMP cyclique dans les cellules pariétales isolées, comme le montrent les valeurs des concentrations intracellulaires de

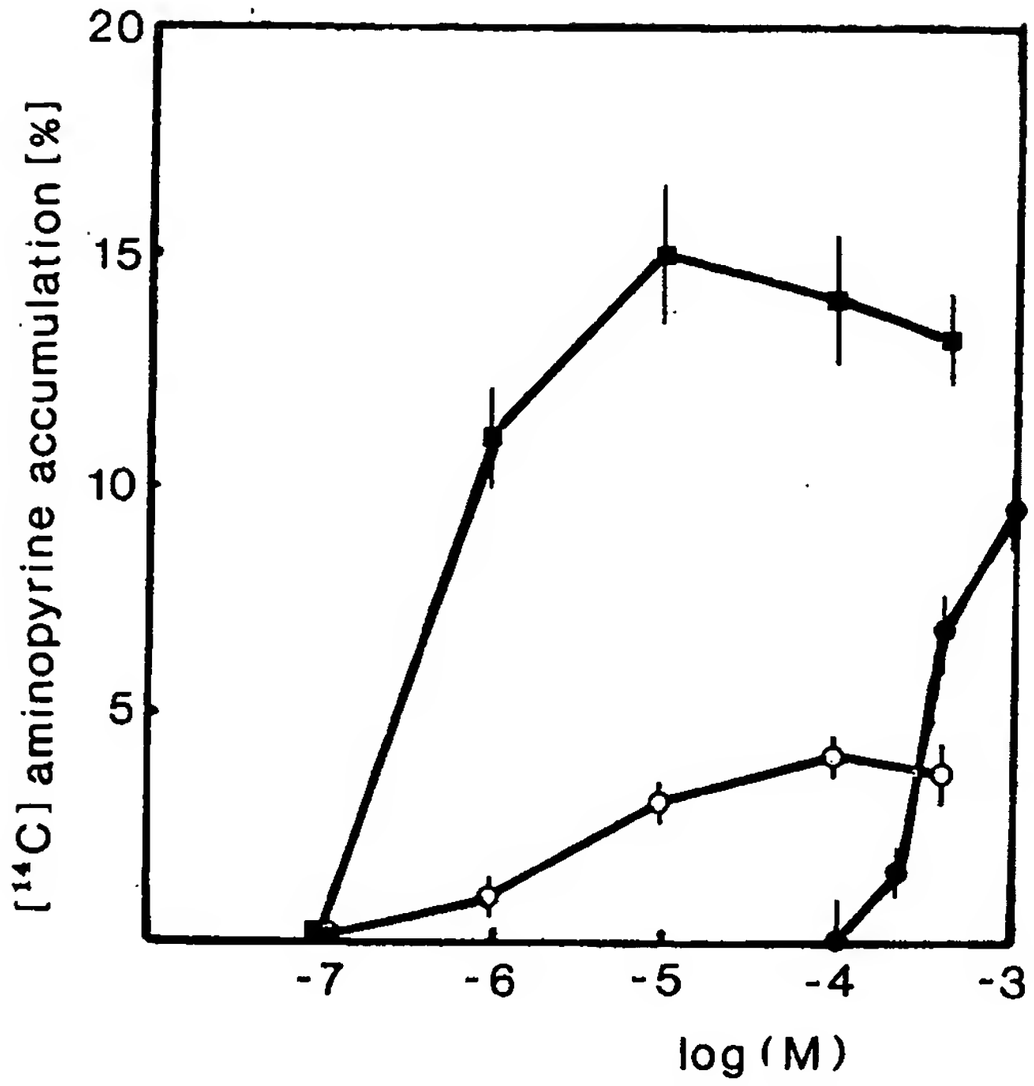


FIG. 2. — Accumulation de [^{14}C]-aminopyrine dans les cellules pariétales isolées.

Les valeurs (± D. S. de 3 déterminations séparées) sont exprimées en % de la radioactivité totale mesurés en présence de différentes concentrations de stimulant histamine (○), forskoline (■), db-cAMP (●).

ce nucléotide en l'absence et en présence de forskoline. L'histamine, à la concentration où elle produit une réponse sécrétoire maximale, entraîne également une élevation du niveau intracellulaire d'AMP cyclique (Fig. 3). Dans les mêmes conditions, la forskoline produit une augmentation 4 fois plus élevée. Toutefois, la valeur maximale ne semble pas atteinte même à la concentration de 0,1 mM de forskoline.

SÉCRÉTION GASTRIQUE ACIDE *in vivo*. — Le modèle du Rat anesthésié de Ghosh et Schild est particulièrement sensible à la gastrine : elle provoque une réponse sécrétoire acide rapide et la réponse maximale est fonction du nombre de cellules pariétales sécrétrices (Fig. 4). Cette réponse sécrétoire à la gastrine est dose-dépendante (DE_{50} : 0,08 nmol/kg).

L'histamine provoque également une réponse sécrétoire dose-dépendante, mais les doses nécessaires pour obtenir cette réponse sont considérablement plus élevées (DE_{50} = 10 µmol/kg), la sécrétion acide gastrique du Rat étant peu sensible à l'histamine. Sur ce modèle pharmacologique, la forskoline entraîne une réponse dose-dépendante dont la puissance (DE_{50} : 100 nmol/kg) est intermédiaire entre celle de l'histamine et celle de la gastrine. Les pentes des courbes dose-réponses sont parallèles et la valeur maximale est la même pour les trois stimulants utilisés.

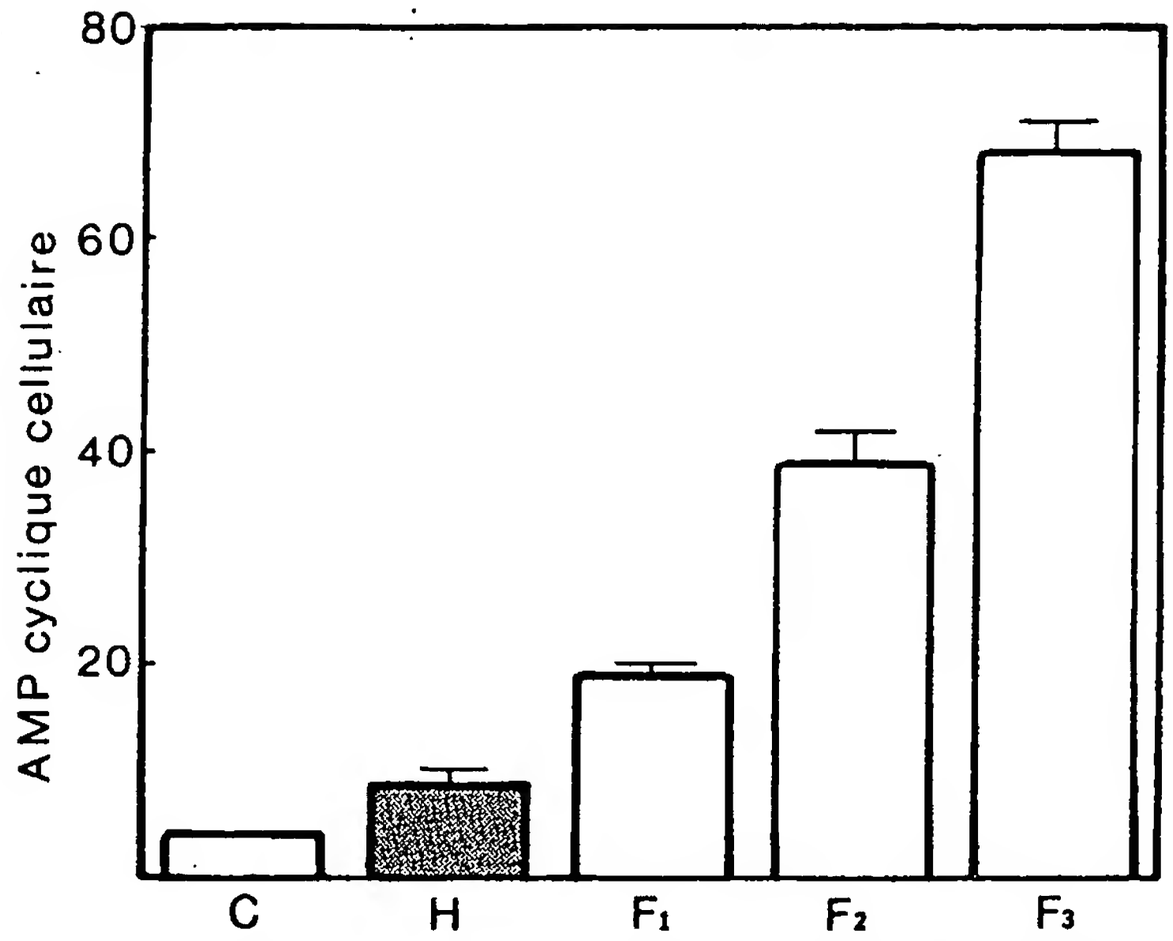


FIG. 3. — Concentrations d'AMP cyclique dans les cellules pariétales isolées.

Mesurées par dosage radioimmunologique après extraction, les concentrations sont exprimées en μmoles d'AMP cyclique par million de cellules pour 5 min d'incubation (± DS de 3 déterminations séparées). Les mesures sont réalisées en présence de 50 μM d'IBMX pour inhiber l'activité phosphodiestérase.

C : cellules F3 sans stimulant; H : cellules en présence d'histamine 10 μM; F1 : cellules en présence de forskoline 1 μM; F2 : cellules en présence de forskoline 10 μM; F3 : cellules en présence de forskoline 100 μM.

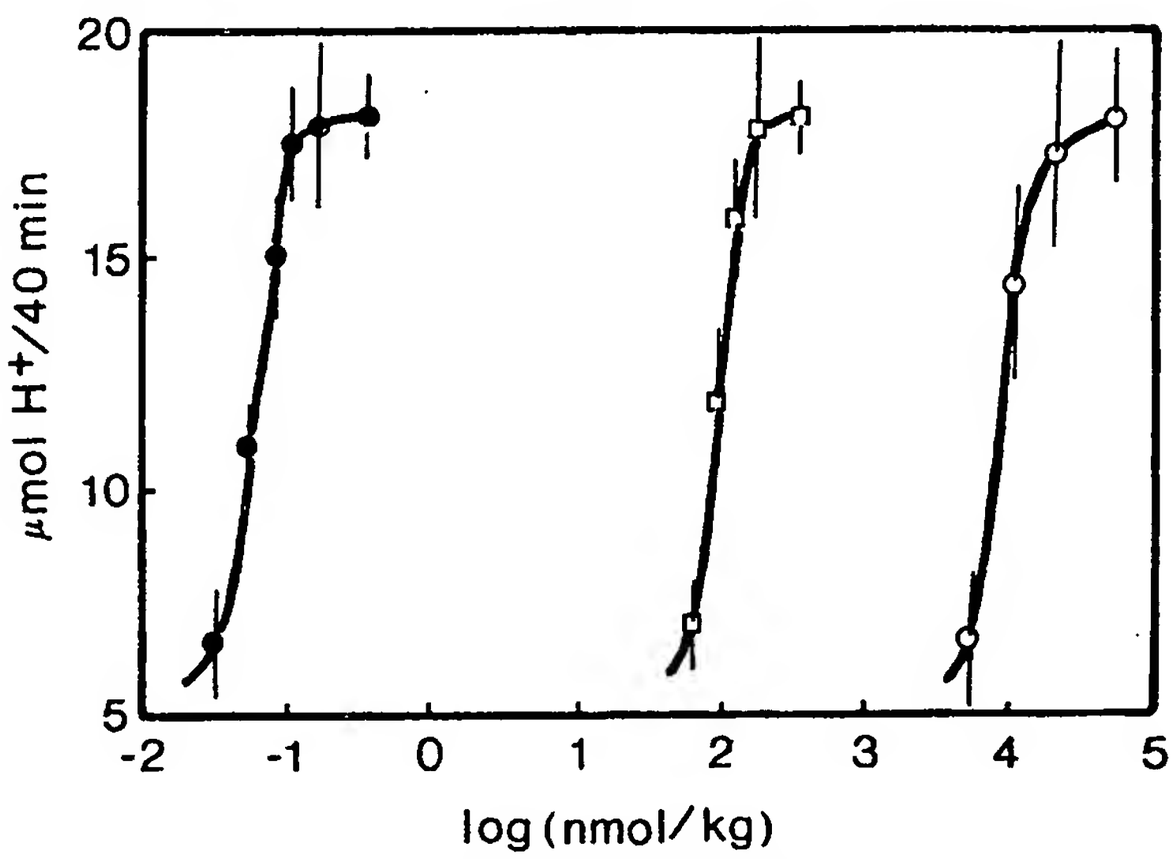


FIG. 4. — Courbes doses-réponses de la sécrétion gastrique acide chez le Rat anesthésié à estomac perfusé.

Les stimulants [gastrine (●), histamine (○) et forskoline (□)] ont été injectés par voie intraveineuse après stabilisation de la sécrétion acide basale. Les expériences ont été réalisées sur 3 animaux par dose de forskoline et sur 15 animaux par dose pour les autres stimulants.

Les valeurs (± DS de 3 ou de 15 déterminations séparées) sont exprimées en μmoles de H^+ sécrétées pour 40 min de stimulation.

La cimétidine (antisécrétoire gastrique utilisé en thérapeutique, à activité anti-histaminique H_2) ne présente pas d'effet inhibiteur majeur sur la sécrétion basale du Rat, ni sur la réponse induite par la forskoline. Par contre, elle effondre totalement la réponse acide induite par l'histamine et à 90 % celle induite par la gastrine (Tableau II).

TABLEAU II. — Sécrétion acide induite par la forskoline chez le Rat anesthésié. Effet de la cimétidine.

Dose ($nmol.kg^{-1}$)	Réponse sécrétoire acide ($\mu mol\ H^+$)	
	seule	+ cimétidine 5 $mg.kg^{-1}$
80	7 ± 1,5	6,6 ± 1,2
120	15,5 ± 2,2	16,5 ± 1,8

La forskoline et la cimétidine ont été injectéés par voie i. v. après stabilisation de la sécrétion acide basale. Les expériences ont été réalisées sur 3 animaux. Les valeurs sont exprimées en µmoles de H^+ sécrétées pour 40 min de stimulation.

Discussion. — Isolée à partir des racines de *Coleus forskohlii*, la forskoline a été caractérisée comme un des diterpènes (isomère C) activateur direct et permanent de la sous-unité catalytique de l'adénylcyclase membranaire d'un certain nombre de tissus. Dans l'estomac, elle stimule cette activité enzymatique adénylcyclase sur des membranes préparées à partir de l'ensemble de la paroi gastrique (4). Sur des préparations de glandes gastriques isolées (6), des résultats comparables ont été obtenus, et montrent une corrélation entre la consommation en oxygène et l'accumulation d'aminopyrine.

Nos résultats obtenus sur cellules pariétales isolées sont en accord avec ceux de la littérature. Cependant, les valeurs de l'accumulation d'aminopyrine et des niveaux d'AMP cyclique obtenus en présence de forskoline sont beaucoup plus élevées (4 fois) que dans le cas des glandes, ce qui traduit la participation directe des cellules pariétales dans la réponse à la forskoline au niveau de la glande fundique. L'observation décrite dans la littérature de l'action inhibitrice de la cimétidine sur la réponse sécrétoire acide des glandes gastriques isolées stimulées par la forskoline (7) suggère que le diterpène potentialise l'action de l'histamine endogène libérée au niveau des glandes. Ce phénomène n'a pas été observé dans nos expériences avec les cellules isolées.

Sur le modèle *in vivo* du Rat anesthésié à estomac perfusé, la forskoline stimule de façon dose-dépendante la sécrétion acide gastrique avec une DE_{50} correspondant sensiblement au 1/100 de la DL_{50} mesurée chez la souris (10 µmol/kg par voie i.v.) (10).

Dans nos expériences, l'effet stimulant de la forskoline sur la sécrétion est observé dans les 5 min qui suivent l'administration i.v., ce qui est compatible avec la demi-vie *in vivo* qui a été évaluée à environ

15 min (11). Si l'on compare la DE_{50} de la forskoline à celle du dibutyryl AMP cyclique pour induire la sécrétion acide, les résultats sont en faveur de la forskoline qui se trouve être, dans ce modèle, environ 100 fois plus puissante (12). Par ailleurs, si l'on estime la volémie du Rat de 300 g à 25 ml, la DE_{50} pour la forskoline correspondrait à une concentration sanguine de 1,2 μM. Cette valeur est du même ordre de grandeur que celle observée pour induire la sécrétion acide des cellules pariétales *in vitro* (CE_{50} : 0,5 μM). Malgré la différence notable dans la taille et la fonction métabolique des compartiments dans lesquels la forskoline a été placée, cette observation semble indiquer qu'elle stimule la sécrétion acide par elle-même, sans biotransformation.

D'autre part, la cimétidine est sans effet sur cette réponse sécrétoire *in vivo*, ce qui laisse suggérer que, dans ce modèle, la participation de l'histamine endogène invoquée à propos de l'effet de la cimétidine sur les glandes gastriques (7) est sans conséquence sur la réponse sécrétoire à la forskoline.

Ainsi, l'association des effets *in vitro* et *in vivo* de la forskoline sur la sécrétion gastrique acide démontre que cette substance se comporte comme un stimulant de la sécrétion gastrique à part entière.

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[Is forskolin a stimulant of gastric secretion?]

[Article in French]

Choquet A, Magous R, Galleyrand JC, Bali JP.

Laboratoire de Biochimie des Membranes, CNRS UPR-41, INSERM U-249, Faculte de Pharmacie, Montpellier.

The diterpene, forskolin, direct activator of the catalytic subunit of the adenylate cyclase from various tissues, also stimulates gastric acid secretion: in vitro, with an isolated parietal cell preparation, forskolin dose-dependently stimulated acid secretion (EC50: 1 microM) (measured by accumulation in the acidic spaces of the weak base [14C]-aminopyrine) and the maximal acid secretory value at 0.1 mM was 4 times higher than that obtained with histamine. Forskolin dramatically increased the production of intracellular cyclic-AMP at a level 4 times higher than that obtained with histamine at the same concentration. In vivo, gastric acid secretion of the rat is dose-dependently increased. The doses required to get a significant response (100 nmol/kg) were 1,000 times higher than those required for gastrin and 100 times lower than those for histamine, but the same maximal value was obtained. Cimetidine did not significantly modified this response. These results demonstrate that, both in vitro and in vivo, forskolin is a potent stimulant for gastric acid secretion.

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SYNAPSE 29:293–298 (1998)

CART Peptides in the Central Control of Feeding and Interactions With Neuropeptide Y

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KEY WORDS NPY; paraventricular nucleus; immunohistochemistry; cocaine

ABSTRACT While CART peptides have been implicated as novel, putative peptide neurotransmitters/cotransmitters, behavioral effects of these peptides have not yet been demonstrated. In this study, we show the first behavioral effect of CART peptides. Icv administration of CART peptide fragments inhibits feeding in rats. Moreover, injection of an antibody to CART peptide 82–103 stimulates feeding, suggesting that endogenous CART peptides exert an inhibitory tone on feeding. Injection of CART peptide 82–103 five min before NPY reduces the increase in feeding caused by injection of NPY alone. Also, in light microscopic immunohistochemical studies, NPY-positive varicosities were observed around CART peptide-positive cell bodies in the paraventricular nucleus of the hypothalamus. These data suggest functional interactions between CART peptides and NPY. These results indicate that CART peptides play a role in the control of food intake by the brain. Synapse 29:293–298, 1998.

INTRODUCTION

CART (cocaine- and amphetamine-regulated transcript) was identified as a mRNA that increased in rat striatum after acute administration of psychostimulants (Douglass et al., 1995). It was subsequently shown to be one of the most abundant regionally specific mRNAs in the hypothalamus (Gautvik et al., 1996), and in situ hybridization studies show it to be discretely localized to specific nuclei throughout the brain (Couceyro et al., 1997; Douglass et al., 1995). The deduced amino acid sequence (Fig.1a) shows that the translated product would have a leader sequence and several pairs of basic amino acids, suggesting that it is processed and secreted. Immunohistochemical results show that CART peptides are in the same neuronal cell groups that contain the CART mRNA (Koylu et al., 1997, 1998) and two CART peptide fragments have been identified in hypothalamic extracts (Spiess et al., 1981). Only partial sequencing of the fragments was carried out and the full sequence of the extracted fragments remains undetermined. At the electron microscopic level, CART peptide immunoreactivity was found in nerve terminals and in large dense core vesicles (Smith et al., 1997). The peptide structure and its localization to certain neurons, terminals, and vesicles suggest that CART peptides represent a novel family of peptide neurotransmitter/cotransmitters. Considering the inhibitory effect of cocaine on food intake, and the presence of CART peptides in brain areas involved in the physiological control of feeding behaviour (Koylu et al., 1997), we chose to investigate the effect of icv CART peptides on food intake. This study shows for the first time that CART peptides have an action in the brain to inhibit food intake and may have both functional and anatomical interactions with the neuropeptide Y (NPY) system.

MATERIALS AND METHODS

Icv Injections and Food Intake Measurement

Male rats (Sprague-Dawley; Charles River Laboratories, Wilmington, MA), average body weight 300 g, were housed singly under a controlled reverse light-dark cycle (12 hours each phase, lights off 10 am) in a temperature-controlled environment. Food and water were available ad libitum. Rats were anaesthetized with an injection of ketamine (Ketaset, Fort Dodge Laboratories, IA; 100 mg/ml; 0.2 ml ip.) followed by a single injection of sodium pentobarbital (Veterinary Laboratories, Inc., KS; 65 mg/ml; 0.2 ml ip.). Once anesthesia had been established, the rat's head was fixed in a stereotaxic frame and a single guide cannula

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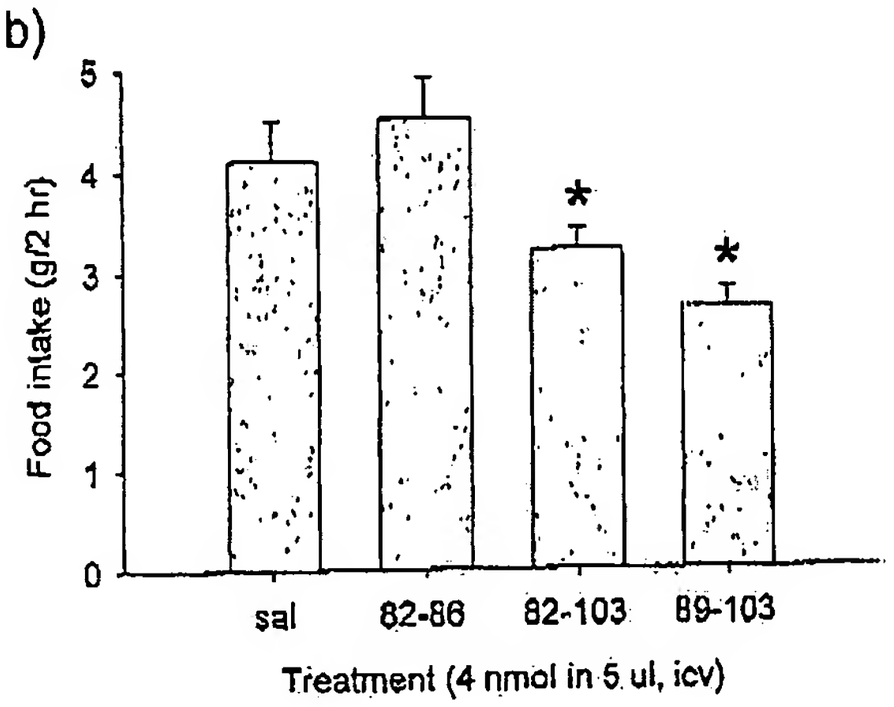
a) CART PROTEIN AMINO ACID SEQUENCE

(1) MESSRLRLLPVLGAALLLLLPLLGAGA (27)
(28) QEDAELQPRALDIYSAVDDASHEKELP (54)
(55) RRQLRAPGAVLQIEALQEVLKKLKS*KR* (81)
(82) IPIY*EKKYGQVPMCDAGEQCAVRK* (105)
(106) GARIGKLCDCPRGTSCNSFLLKCL (129)



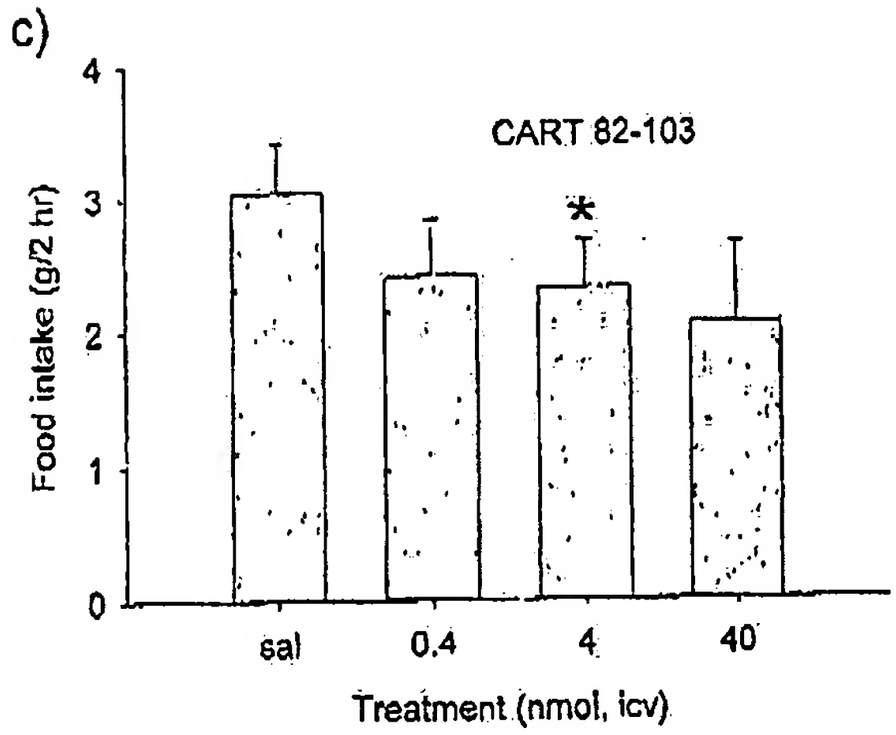


Fig. 1. Effect of icv injection of CART peptide fragments on dark phase food intake in the rat. a: CART peptide sequence. The amino acid sequence of CART peptide is shown. Numbers in parenthesis indicate the amino acid residue. The portion of the peptide investigated in the text is underlined and fragments studied are found between potential processing sites shown in italics. b: Effects of CART peptides 82–86, 82–103, and 89–103 (4 nmol/5 μl; icv) on dark-phase food intake (g/2 hour) compared to 0.9 % saline (sal) (n = 5–13). c: Dose response curve for CART 82–103 (0.4, 4, 40 nmol/5 μl; icv) on dark-phase food intake (g/2 hour) (n = 7). See text for details. Statistical analysis of data shown in b and c was by paired *t*-test. *Statistically significant differences from sal group, $P < 0.05$.

(22 gauge; Plastics One Inc., Roanoke, VA) was implanted just above the lateral ventricle (AP +0.8, L 1.4, V −3.0). Three stainless steel screws were attached to the skull and the cannula was fixed in place using dental cement applied around the screws and the cannula. A 26 gauge stainless steel flush-fitting stylet (Plastics One Inc.) was inserted into the cannula to prevent blockage. Animals were allowed at least 5 days to recover from surgery before being used in the experimental procedure. Forty-eight hours prior to the experimental procedure, all animals were injected icv with human angiotensin II (150 ng per rat; Sigma, St. Louis, MO) and only those that demonstrated a marked drinking response, within 10 minutes of injection, were included in the studies. Infusions were made on conscious rats in their home cage. The stylet was removed and a 26 gauge steel cannula projecting 1 mm below the guide cannulae was inserted. The injection cannula (Plastics One Inc.) was connected via tubing (Intramedic PE20 polyethylene tubing; Clay Adams) to a 10 ml Hamilton (Reno, NV) syringe driven by a Hamilton Apparatus 22 infusion pump. The syringe and tubing were filled with sterile 0.9% NaCl and a small air bubble was used to separate the infused solution from the saline and also acted as a convenient index of a successful infusion. All peptides were dissolved in 0.9% saline at concentrations requiring a 5 μl icv injection. All solutions were infused over a 2-minute period and the infusion cannulae were left in place for a further minute. All animal procedures were performed according to rules established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Institutional Animal Care and Use Committee of Emory University.

Following infusion, the animal was placed in the test arena (home cage without bedding) along with a preweighed amount of rat chow and free access to water. At the end of a 2-hour test period, all remaining food was collected and reweighed to determine total food intake. In experiments investigating the effect of CART peptides (Neurocrine Biosciences Inc., San Diego, CA; Multiple Peptide Systems, San Diego, CA) or antisera to CART 82–103 (Cocalico Inc., Reamstown, PA) on dark-phase feeding, icv injections were made 10 minutes prior to lights out. Polyclonal antisera were raised against CART 82–103 and were specific to that peptide (Koylu et al., 1997, 1998). NPY-induced food intake experiments were carried out in the light phase with an icv injection of CART peptide given 5 minutes before icv NPY injection. All experiments were conducted in a crossover design with each animal receiving an icv injection of peptide on test day 1 followed by saline on test day 2, or vice versa. In this way, each animal served as its own control and the data facilitated the use of more powerful paired statistical methods. A period of at least 48 hours was allowed between test days. All data were grouped by treatment, mean and standard errors of the mean were calculated, and significant differences between groups were assessed using a paired *t*-test or in the case of multiple treatments an ANOVA followed by Tukey's post-hoc test.

Tissue Preparation for Immunobistochemistry

Three adult male Sprague-Dawley rats (200–250 g) were deeply anesthetized with an overdose of pentobarbital. The animals were then perfusion-fixed with a Ringer solution and a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (0.1M, pH 7.4). All animal procedures were performed according to rules established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Institutional Animal Care and Use Committee of Emory University. The brains were cut in 60-μm-thick sections with a vibrating microtome and processed for immunohistochemistry at the light microscopic level.

Simultaneous Immunohistochemical Localization of CART and NPY

Sections were pretreated with sodium borohydride (1% in phosphate buffered saline [PBS], 0.01M, pH 7.4) for 20 minutes, and preincubated with 1% normal goat serum (NGS; Vector Laboratories, Burlingame, CA), 1% bovine serum albumin (BSA; Sigma), and 0.4% Triton X-100 in PBS for 1 hour. They were then incubated at 4°C for 48 hours in the primary antibody solution. The CART peptide antibodies were prepared by Cocalico Inc. (Reamstown, PA) using standard procedures. The preparation and tests for the specificity of the CART antisera are described elsewhere; only the immunizing peptide blocked immunohistochemical staining and preimmune serum gave no significant staining (Koylu et al., 1997, 1998). In the present study, we used a rabbit polyclonal antiserum (1:5,000 in 1% NGS, 1% BSA, 0.4% Triton X-100 in PBS) directed against the deduced peptide segment CART 106–129 from rat CART. After the primary incubation, the sections were incubated for 90 minutes at room temperature with biotinylated goat anti rabbit IgGs (Vector Laboratories; 1:200 dilution) in PBS containing 1% BSA, 1% NGS, and 0.4% Triton X-100. The sections were then washed three times (10 minutes each) in PBS before being placed in a solution containing avidin-biotin peroxidase complex (ABC; Vector Laboratories; 1:100 dilution) and 1% BSA in PBS. They were then washed in PBS and TRIS buffer (TBS; 0.05M, pH 7.6) prior to a 10-minute incubation in 3,3' diaminobenzidine (DAB; 0.025%), ammonium nickel sulfate (Ni-DAB; Fischer Scientific, Fair Lawn, NJ; 0.35%), and 0.0006% hydrogen peroxide to localize the immunoreactivity. The reaction was stopped by repeated washes in PBS. The Ni-DAB-stained sections were then incubated overnight at 4°C in a sheep anti- NPY antibody solution (Chemicon, Temecula, CA; 1:3,500 dilution), 1% NGS, 1% BSA, and 0.4% Triton X-100. After three washes (10 minutes each) in PBS, the sections were incubated for 90 minutes at room temperature with biotinylated donkey anti sheep IgGs (Sigma; 1:50 dilution), 1% BSA, 1% NG,S,and 0.4% Triton X-100. Following three washes (10 minutes each) in PBS, the sections were placed in a solution containing ABC (1:100 dilution) and 1% BSA in PBS for 90 minutes at room temperature. Washes in PBS and TRIS buffer preceded incubation in DAB (0.025%), 0.01 M Imidazole, and hydrogen peroxide (0.006%) for 10 minutes (Wouterlood et al., 1987). The sections were then mounted on chrome-alum/gelatin-coated slides, dehydrated, and a coverslip was applied with Permount.

Control experiments were carried out for the double labeling studies. Controls included sections processed for CART or NPY immunostaining alone or sections processed for the double immunostaining except that each of the primary antibodies was omitted in turn from the incubation solutions. The distribution and quality of the immunostaining for CART and NPY in the single stained sections were comparable to that obtained after double immunostaining, which indicates that there was no loss of immunoreactivity induced by the double labeling procedures. After omission of each primary antisera, sections were stained only with Ni-DAB when the NPY antibodies were omitted or only with Ni-DAB when the CART antibodies were omitted. The pattern of staining for each antisera corresponded to that seen in single stained sections irrespective of the chromogen that has been used. This indicates that there was no nonspecific staining generated by potential cross-reactions between the two chromogens.

RESULTS

The CART peptide fragments (Fig. 1a) chosen for study (82–86, 82–103, and 89–103) were based on possible processing patterns, immunoreactivity data (Koylu et al., 1997, 1998), and literature findings (Spiess et al., 1981).

Peptide fragments were injected into the lateral ventricle of male rats via a chronically implanted cannula. A pump was used to ensure smooth and reproducible delivery of the peptides (2.5 μl/min). Initially, various CART peptides were injected in equimolar quantities, and food intake in the first 2 hours of the dark phase was measured (Fig. 1b). CART 82–103 (4 nmol/5 μl) and 89–103 (4 nmol/5 μl) reduced food intake by 26 and 40%, respectively, compared to injection of saline (5 μl); the effect of CART 82–86 (4 nmol/5 μl) was not different from saline. Icv injection of 0.4, 4, and 40 nmol of CART 82–103 resulted in a dose-dependent trend in the inhibition of food intake (Fig. 1c).

Icv injection of polyclonal antisera raised against 82–103 produced a volume dependent (1, 3, 5, 10 μl) increase in food intake compared to injection of preimmune sera (Fig. 2a,b). The effect of preimmune serum was not statistically different from saline injections (data not shown). The finding that the effect of the antisera was stimulatory and opposite to the effects of the peptides strongly suggested that the observed

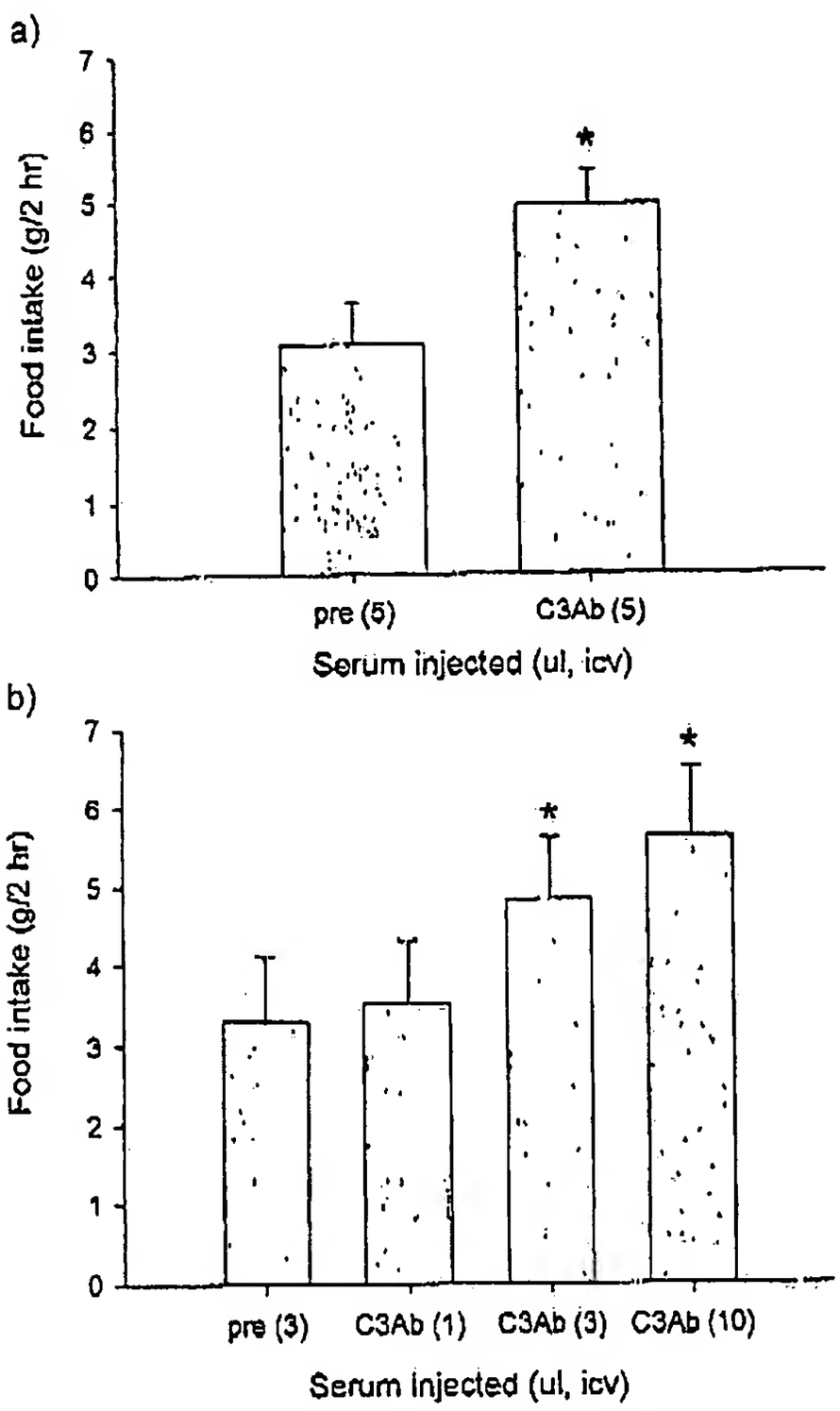


Fig. 2. Effects of icv injection of preimmune (pre) and immune serum (C3Ab) (to CART 82–103) on dark-phase food intake (g/2 hour). a: Icv injection of preimmune (5 µl) and immune (5 µl) serum (n = 11). b: Volume-response to icv injection of immune serum (1, 3, 10 µl) compared to preimmune serum (pre, 3 µl) (n = 4). Statistical analysis of data shown in a was by paired *t*-test. *Statistically significant difference from pre group, $P < 0.05$. Statistical analysis of data shown in b was by one-way repeated measures ANOVA ($F(3,13) = 21.8$, $P < 0.001$) followed by a post hoc Tukey test. *$P < 0.05$ compared to pre group.

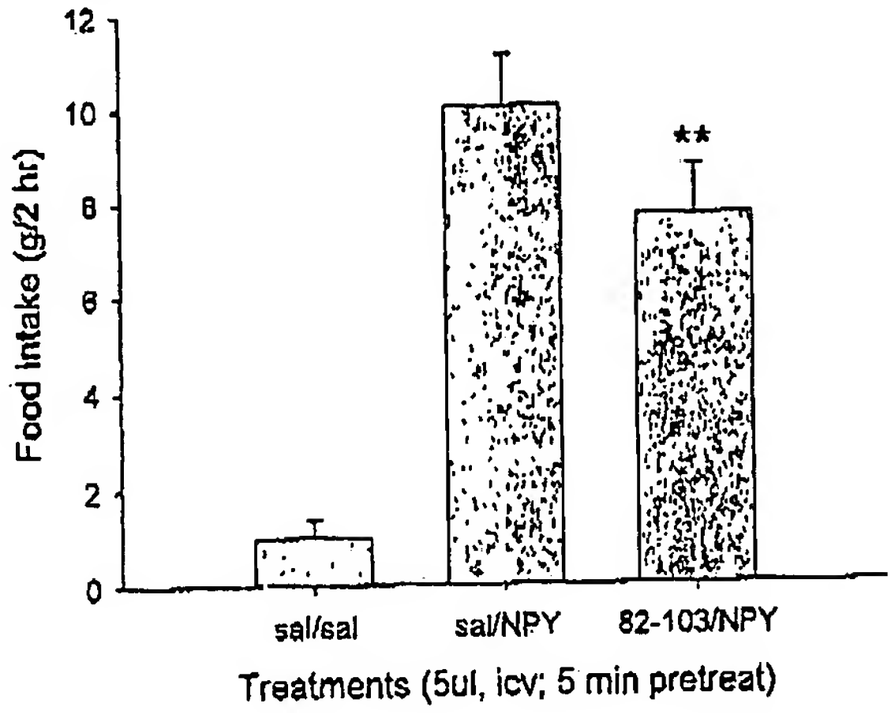


Fig. 3. Effects of a 5-minute pretreatment with 0.9 % saline (sal, 5 µl; icv) or CART 82–103 (4 nmol/5 µl; icv) on NPY-induced food intake (g/2 hour) in the rat (n = 6). Statistical analysis of data was by paired *t*-test. **Statistically significant difference from sal/NPY group, $P < 0.01$.

effects of the peptides were not nonspecific and reflected the activity of endogenous CART peptides. Further, the stimulation caused by the antisera suggests that endogenous CART peptides exert an inhibitory tone on feeding in the first 2 hours of the dark phase.

Neuropeptide Y (NPY) is the most powerful stimulant of feeding known (Clark et al., 1984; Lambert et al., 1993a) and has been shown to functionally interact with other hypothalamic neuropeptides, such as dynorphin (Lambert et al., 1993b) and GLP-1 7–36NH_2 (Turton et al., 1996) in the central control of food intake. CART peptide-immunoreactivity is found in hypothalamic nuclei containing NPY (Koylu et al., 1997), and it is generally accepted that central satiety factors would interact with the NPY system to achieve a fine control on feeding behavior. Therefore, we sought to determine if both functional and anatomical interactions between CART peptides and the NPY system exist. NPY is well established as a powerful orexigenic agent and we show here that icv injection of NPY (2.4 nmol/5 µl) dramatically increased 2-hourly food intake (10 ± 1 g) compared to injection of saline (1.0 ± 0.4 g) (Fig. 3). Interestingly, icv injection of CART 82–103 (4 nmol/5 µl) 5 minutes before injection of NPY (2.4 nmol/5 µl) significantly attenuated the feeding response to NPY (7.7 ± 1.1 g) (Fig. 3).

Having demonstrated the effect on feeding of an interaction between CART 82–103 and NPY, we conducted immunohistochemical studies in the rat paraventricular nucleus of the hypothalamus (PVN), at the light microscopic level, to test if there was an anatomical interaction between NPY and CART peptide-immunoreactive elements. An antibody against CART peptide 106–129 was used for the immunohistochemical localization studies. The distribution of CART peptide immunoreactivity at the level of the PVN was similar with either CART 82–103 or CART 106–129 antisera (Koylu et al., 1997). However, CART 106–129 antisera gave more consistent results in the double labeling studies with NPY presented here. In double immunostained sections, NPY-containing varicosities were found to form dense pericellular baskets around the perikaya of CART peptide-immunoreactive neurons in the parvicellular region of the PVN (Fig. 4b,c). Electron microscopic studies are underway to verify the possibility that NPY-immunoreactive terminals establish direct synaptic contacts with CART peptide-containing neurons in the rat and monkey PVN. Prelimi-

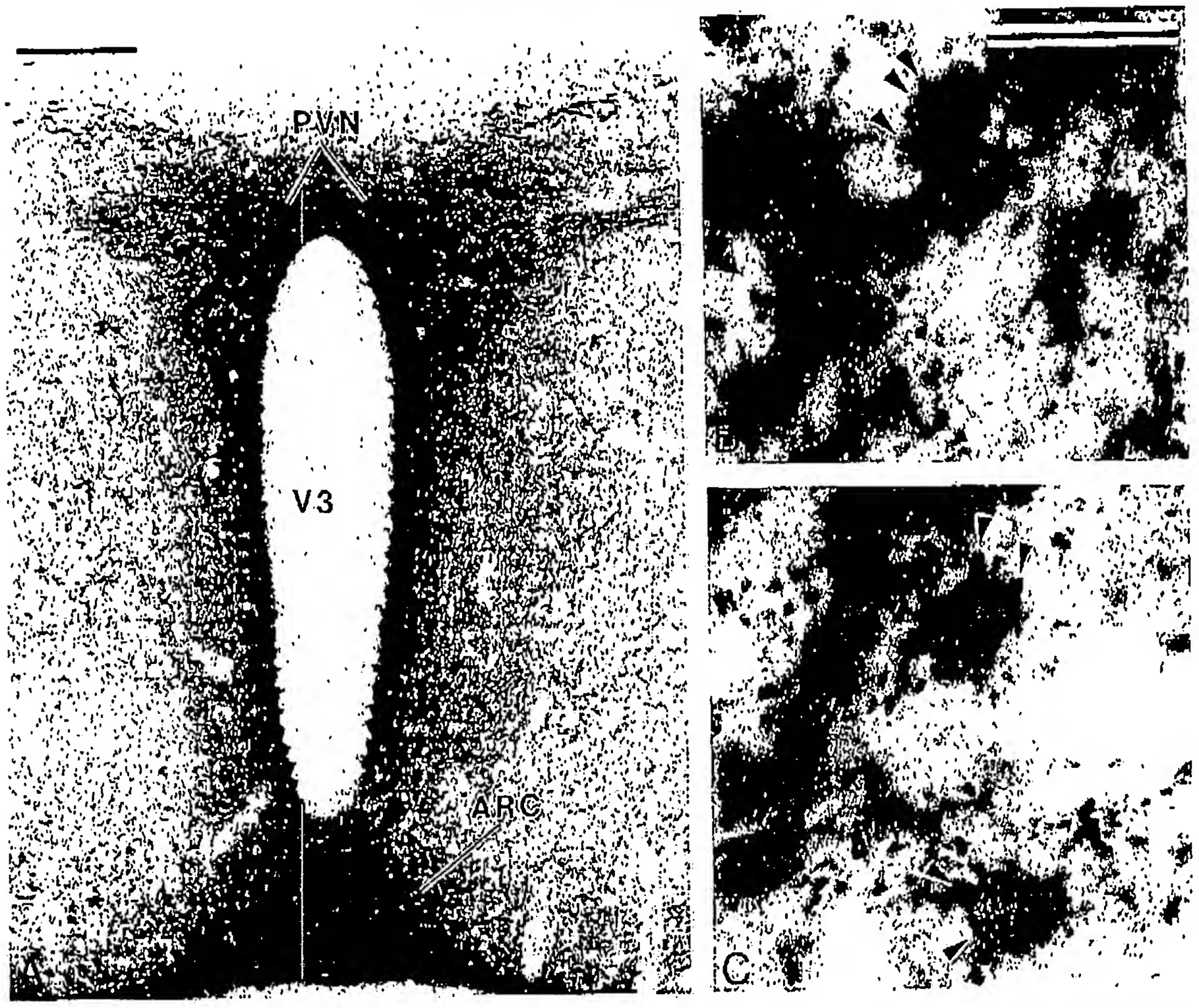


Fig. 4. Light microscopic immunohistochemical localization of CART peptides and NPY in the paraventricular nucleus of the hypothalamus of the rat. a: Double immunostained sections for CART peptide immunoreactivity (NiDAB, blue) and NPY immunoreactivity (DAB, brown) in the paraventricular nucleus of the hypothalamus in the rat (scale bar = 250 μm). b,c: High-power views of CART peptide-immunoreactive cell bodies surrounded by NPY-containing varicosities (arrowheads) in the PVN (scale bar = 20 μm). V3 = third ventricle; ARC = arcuate nucleus.

nary electron microscopic data show CART peptide-positive cell bodies and NPY-positive elements in apposition (data not shown).

DISCUSSION

CART peptides are putative neurotransmitter/co-transmitters (Couceyro et al., in press; Douglass et al., 1995; Koylu et al., 1997, 1998; Smith et al., 1997). The data shown here are the first to show any behavioral effects of CART peptides. We have utilized peptides in the region of CART 82–103 that are possible processed fragments of CART peptides found in the hypothalamus (Koylu et al., 1997; Spiess et al., 1981). CART 82–103 and 89–103 show effects on feeding while CART 82–86 did not. While we cannot be sure that these fragments are produced in the brain, they are likely to be active at endogenous receptors for CART peptides. Certainly, the effects of antiserum to CART 82–103, but not preimmune serum, indicate tonic, endogenous activity of a peptide that is at least similar.

An effect of CART peptide 82–103 on food intake after icv injection is consistent with CART peptide localization in the hypothalamus. A peptide containing the CART fragment 82–103 at its N-terminal has been identified in the hypothalamus (Speiss et al., 1981) and we have identified immunoreactivity to CART 82–103 in hypothalamic nuclei involved in the control of feeding and body weight (Koylu et al., 1997). In addition to the effect of CART peptides in inhibiting feeding, we also show an increase in food intake following icv injection of a polyclonal antisera raised against CART peptide 82–103. These data are critical in establishing a physiological role for endogenous CART peptides in the control of food intake. Neuropeptide Y is established

as an important central mediator of food intake (Clark et al., 1984; Lambert et al., 1993a,b) and has a physiological role in the delicate balance between calorie intake and energy expenditure, which determines body weight (Leven and Routh, 1996; Wang et al., 1997). It seems probable that any central neuropeptide involved in reduction of food intake would have an interaction with NPY in order to achieve a fine control of feeding. The data here further implicate CART peptides in the complex central control of food intake by showing both a behavioral and anatomical interaction with NPY.

In summary, these data identify CART peptides as novel central components in the control of food intake and suggest a role for endogenous CART peptides as physiological satiety factors. The apparent interaction with NPY indicates that CART peptides can influence the known hypothalamic circuitry that regulates feeding. Studies elucidating the mechanisms by which CART peptides affect feeding are ongoing. Furthermore, continuing studies are demonstrating that other physiological processes are mediated by CART peptides. These findings have been presented in abstract form (Lambert et al., 1997).

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Promotion of Forskolin-Induced Long-Term Potentiation of Synaptic Transmission by Caffeine in Area CA1 of the Rat Hippocampus

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Abstract

Caffeine which is present in soft drinks has been shown to increase alertness and allays drowsiness and fatigue. The aim of this study is to investigate whether caffeine could produce a long-term effect on the synaptic transmission using extracellular recording technique in the hippocampal slices. Bath application of caffeine (100 μM) reversibly increased the slope of field excitatory postsynaptic potential (fEPSP). Forskolin (25 μM) by its own did not affect the fEPSP significantly. However, in the presence of caffeine, forskolin induced a long-term potentiation (LTP) of fEPSP. Enprofylline which has been shown to exhibit some actions like caffeine but with a low adenosine antagonistic potency did not affect the normal synaptic transmission or the effect of forskolin at a lower concentration (10 μM). However, when the concentrations were increased to 20 and 50 μM, enprofylline significantly enhanced the fEPSP slope and promoted forskolin-induced LTP. The parallel increase of fEPSP and promotion of LTP observed with enprofylline suggests that adenosine A_1 antagonism is the primary mechanism behind caffeine's effect. This hypothesis was further strengthened by the finding that promotion of forskolin-induced LTP was mimicked by the non-xanthine adenosine antagonist 9-chloro-2-(furyl)[1,2,4]triazolo [1,5-c]quinazolin-5-amine (CGS 15943). The promotion of forskolin-induced LTP provides a cellular basis behind caffeine's increase in capacity for sustained intellectual performance.

Key Words: caffeine, long-term potentiation, forskolin, adenosine, hippocampus, c-AMP

Introduction

Caffeine which is present in soft drinks, coffee, tea, cocoa and chocolate is the most widely used social drug in the world. The ingestion of 85 to 250 mg of caffeine, the amount contained in 1 to 3 cups of coffee, increases alertness and allays drowsiness and fatigue. As the dose of caffeine increased, signs of progressive CNS stimulation appeared, including nervousness, anxiety, restlessness, insomnia, tremors and hyperesthesia. In more severe cases, focal and generalized convulsion may occur (4).

At the cellular level, caffeine has been shown to inhibit cyclic nucleotide phosphodiesterase (23), to antagonize adenosine receptors (8,14) and to interfere with the uptake and storage of Ca^{++} by the sarcoplasmic reticulum in striated muscle (18). However, it is still not known which of these effects is most relevant to its enhancement of cognitive function. Long-term potentiation (LTP) of synaptic transmission is a cellular process thought to underlie some forms of learning and memory (5). In hippocampal CA1 neurons, increase in presynaptic cAMP level by activation of β-adrenergic receptors or adenylyl cyclase only caused a transient enhancement of glutamate release and LTP was not observed consistently. However, when adenosine A_1 receptors were blocked or the metabolism of cAMP was disrupted, activation of adenylyl cyclase by forskolin induced LTP (17). These results suggest that it is adenosine which acts on adenosine A_1 receptors to mask forskolin-induced LTP. In this study, we test

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the hypothesis that if caffeine can promote forskolin-induced LTP in the hippocampal CA1 neurons and whether this effect is due to blockade of adenosine A_1 receptor.

Materials and Methods

Male Sprague-Dawley rats of 5- to 7-week-old were decapitated and the brains rapidly removed from the skull. Coronal slices of 400-450 µm thick were cut and the appropriate slices were placed in a beaker of artificial cerebrospinal fluid (ACSF). The ACSF was bubbled continuously with 95%O_2-5%CO_2 to maintain the proper pH (7.3-7.5). The composition of the ACSF solution was (in mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2 and glucose 11. The slices were kept at room temperature for at least one hour before being transferred to the recording chamber where it was held submerged between two nylon nets and maintained at 32±1°C.

Extracellular recordings of fEPSPs were obtained from stratum radiatum using microelectrodes filled with 3 M NaCl (3-8 MΩ). A bipolar stimulating electrode was placed in stratum radiatum for stimulation of Schaffer collateral/commissural pathway. The stimulus duration was 150 µs and the stimulus intensity was adjusted individually for each experiment to produce fEPSP which were ~30-40% of the maximal responses that could be evoked. Experimental treatments were not initiated until the response had been stable for at least 20 min. The strength of synaptic transmission was quantified by measuring the initial slope of the fEPSP. The fEPSP slopes were measured by linear regression of their initial rising phases, usually during the first 0.4-0.6 ms after their onset. Onset was taken after the afferent volley.

Data were analyzed using pClamp data acquisition and analysis softward (Axon Ins., Foster City, CA, USA) running on a PC586 computer. All data were expressed as mean±S.E.M. Statistical analysis was performed using Student's *t*-test and a *p* value of less than 0.05 was considered to be statistically significant. Forskolin and caffeine were purchased from Sigma Chemicals (St. Louis, MO, USA), and other drugs were obtained from Research Biochemicals International (Natick, MA, USA).

Results

The effect of caffeine on the fEPSP slope as a function of time is illustrated in Figure 1. After the evoked responses were stable for 20-30 min, caffeine was bath applied. At a concentration of 100 µM, caffeine increased the fEPSP slope by an average of 86±14% (n=7, $p<0.001$). The effect of caffeine was

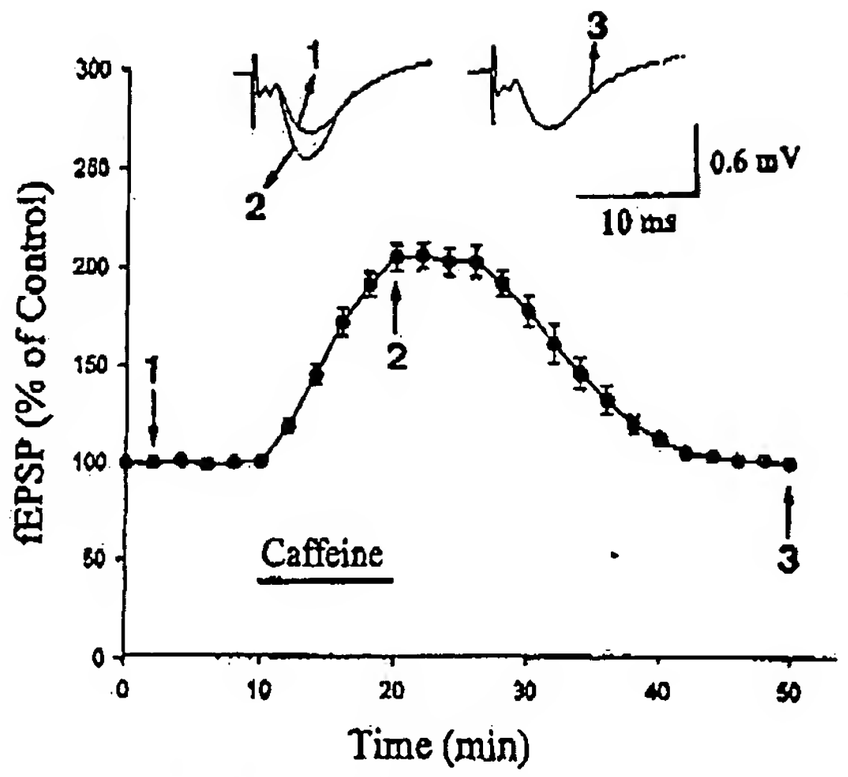

Fig. 1. Reversible enhancement of fEPSP by caffeine. The slope of fEPSP was plotted as a function of time. Inset shows the records taken before and during the application of caffeine (100 µM).

reversible which returned to baseline level within 30 min of washing with control ACSF.

Figure 2 shows that forskolin at the concentration of 25 µM had no significant effect on the fEPSP, a result consistent with previous reports (9). However, in the presence of caffeine, forskolin (25 µM) induced LTP of the fEPSP slope in 8 out of 9 slices tested. The slope of fEPSP remained 167±12% of baseline (n=8, $p<0.01$) 60 min after washout of forskolin.

Caffeine could exert its effect by inhibiting phosphodiesterase (23), blocking adenosine A_1 receptors (8. 14) and releasing Ca^{++} from intracellular stores (13, 18). To determine which effect accounts for the promotion of forskolin-induced LTP, we made use of enprofylline which has been shown to exhibit some actions like caffeine but with a low adenosine antagonistic potency (11, 19). Figure 3 shows that low concentration (10 µM) of enprofylline did not either affect the normal fEPSP (108±4% of baseline, n=7) or the effect of forskolin (108±10% of control, n=7). However, when the concentrations were increased to 20 and 50 µM, enprofylline significantly increased the fEPSP by 46±15% (n=7, $p<0.01$) and 80±7% (n=7, $p<0.001$) respectively, and subsequently promoted the forskolin-induced LTP. The slopes of fEPSP were 128Ñb11% (n=7, $p<0.01$) and 184±11% (n=7, $p<0.001$) of control 60 min following washout of the drugs (Fig. 3).

We speculated that the enhancement of fEPSP and promotion of forskolin-induced LTP by enprofylline was due to its antagonism of adenosine A_1 receptor by testing the effect of enprofylline on the A_1 receptor-induced synaptic depression. 2-chloroadenosine (2-CA), a selective adenosine A_1

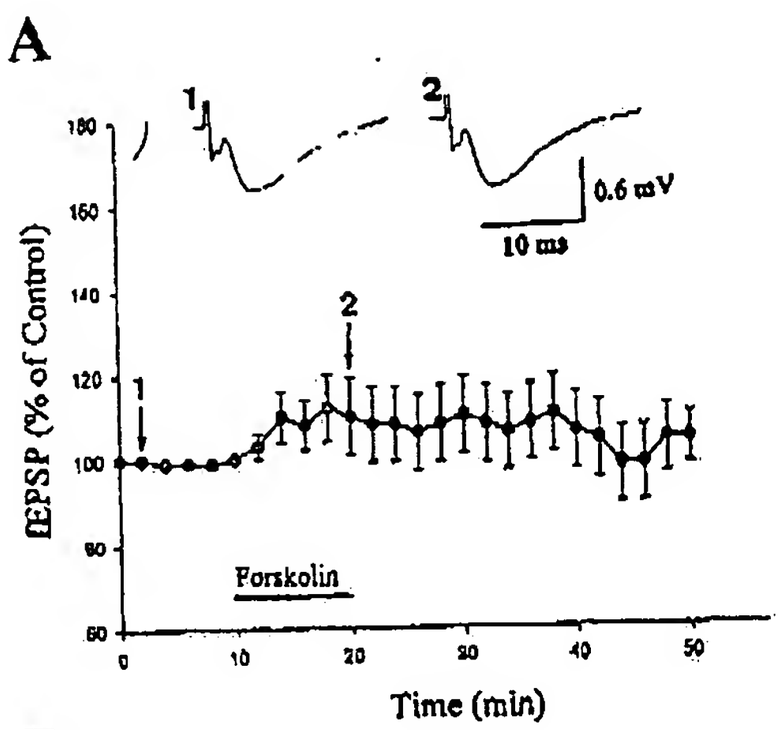

Fig. 2. Caffeine promotes forskolin-induced LTP. A, Effect of forskolin on the fEPSP. The slope of fEPSP was plotted as a function of time. Inset shows the records taken before and during the application of forskolin (25 μM). B, Application of forskolin in the presence of caffeine resulted in a long-term enhancement of fEPSP. Superfusion of caffeine (100 μM) increased the fEPSP slope. Subsequent addition of forskolin (25 μM) in the presence of caffeine resulted in LTP. Inset is superimposed traces taken at different times as indicated.

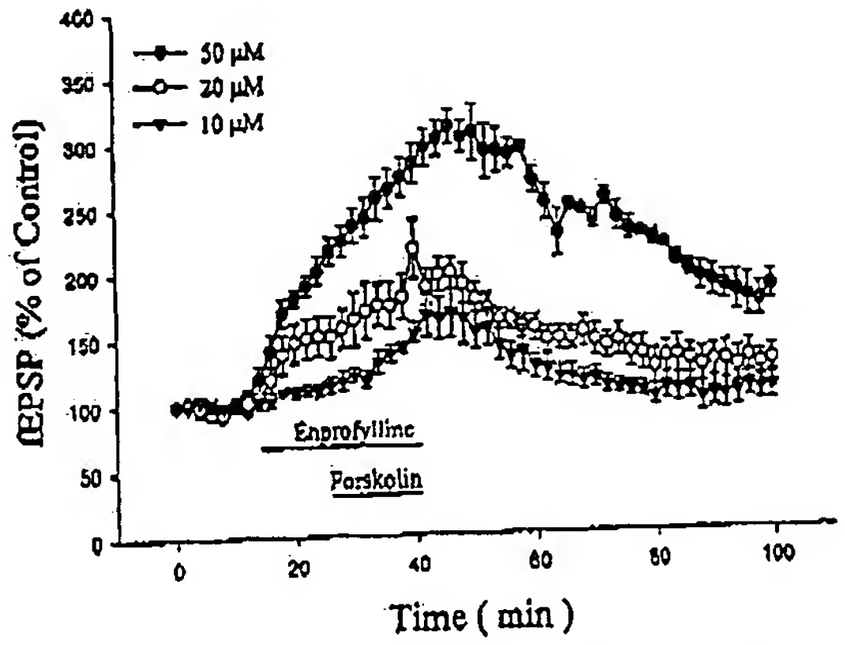

Fig. 3. Concentration-dependent effect of enprofylline on the fEPSP. Application of enprofylline of increasing concentrations enhanced the fEPSP slope and promoted the forskolin-induced LTP.

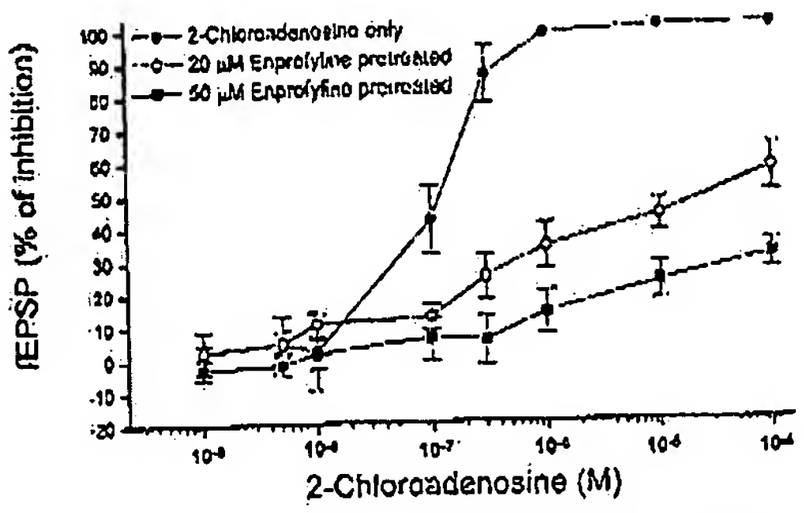

Fig. 4. Antagonism of 2-CA-mediated inhibition of fEPSP by enprofylline. The percent inhibition of fEPSP was plotted against the concentrations of 2-CA in the absence and the presence of enprofylline.

agonist caused an inhibition of fEPSPs. The effect reached a steady state within 5 min and readily reversed when the 2-CA was washed from the tissue. The inhibition of fEPSPs was concentration-dependent and a 50% inhibition (EC_{50}) was about 100 nM. Figure 4 shows that the concentration-response curve for the inhibitory effect of 2-CA was shifed to the right by enprofylline. The fEPSP slope was reduced by 98.4±1.5% (n=6) in the presence of 1 μM 2-CA. Same concentration of 2-CA only reduced the fEPSP by 33.5±7.3% (n=6) and 13.3±6.8% when slices were pretreated with 20 and 50 μM of enprofylline respectively (Fig. 4). There is a significant difference ($p<0.01$) between control and those enprofylline-pretreated slices.

CGS-15943, 9-chloro-2-(furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine, is a novel nonxanthine adenosine antagonist without exhibiting inhibitory activity on the phosphodiesterases (6, 7). Figure 5 is a summary of 6-7 experiments showing that superfusion of 5, 50 and 100 μM of CGS-15943 increased the slope of fEPSPs by 6.1±5.4, 11.6±4.5 and 54.5±3.6% respectively. Furthermore, in the presence of CGS-15943 (100 μM), forskolin induced LTP. The fEPSP slope was 128±3% (n=7, $p<0.001$) of control 50 min after washout of the drugs (Fig. 5B).

To investigate whether forskolin+caffeine-induced LTP is mediated through activation of cAMP-dependent protein kinase (PKA), we performed experiments with the specific PKA regulatory site antagonist, Rp-cyclic adenosine 3',5'-monophosphothioate (Rp-cAMPS). Slices were presoaked initially in 100 μM solution of Rp-cAMPS in the incubation beaker and then transferred into the

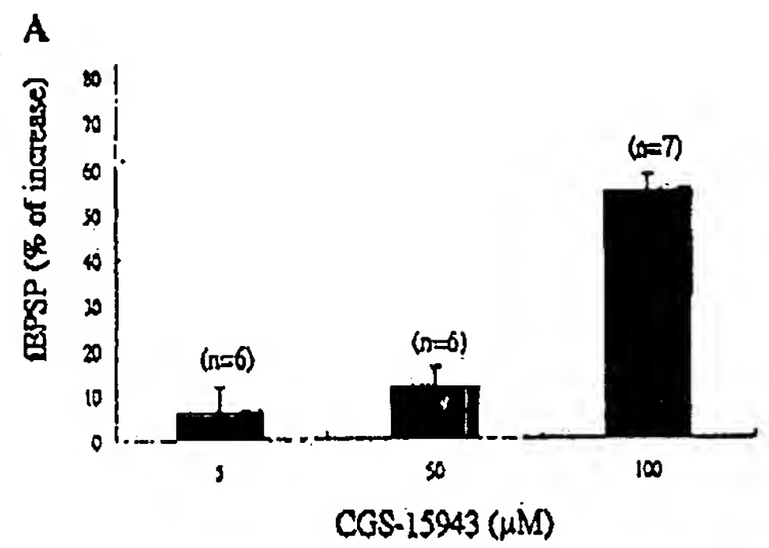

Fig. 5. Pretreatment with nonxanthine antagonist CGS-15943 promotes forskolin-induced LTP. A, Concentration-dependent enhancement of fEPSP by CGS-15943. B, Effects of CGS-15943 on the fEPSP and forskolin-induced LTP.

recording chamber where the concentration was maintained at 25 μM. As shown in figure 6, forskolin+caffeine-induced LTP was blocked (106±6% of control, n=6, $p<0.01$ unpaired t-test).

Discussion

Pharmacologically, it is well known that xanthine-like compounds have several profound central effects: proconvulsant, anxiogenic, antidepressant and CNS stimulatory actions. The results of this study add an additional, long-term effect of caffeine in enhancing the cognitive performance, provided that LTP represents a mechanism for learning and memory (5). At the cellular level, caffeine has three distinct effects: inhibition of phosphodiesterase (23), blockade of adenosine receptors (8, 14) and release of Ca^{++} from intracellular stores (16, 18). Since caffeine requires concentrations in the millimolar ranges (1-10 mM) for significant Ca^{++} release (16, 18), it is unlikely that induction of Ca^{++} release is the mechanism behind caffeine's enhancement of synaptic transmission and promotion of forskolin-induced LTP.

To differentiate between phosphodiesterase

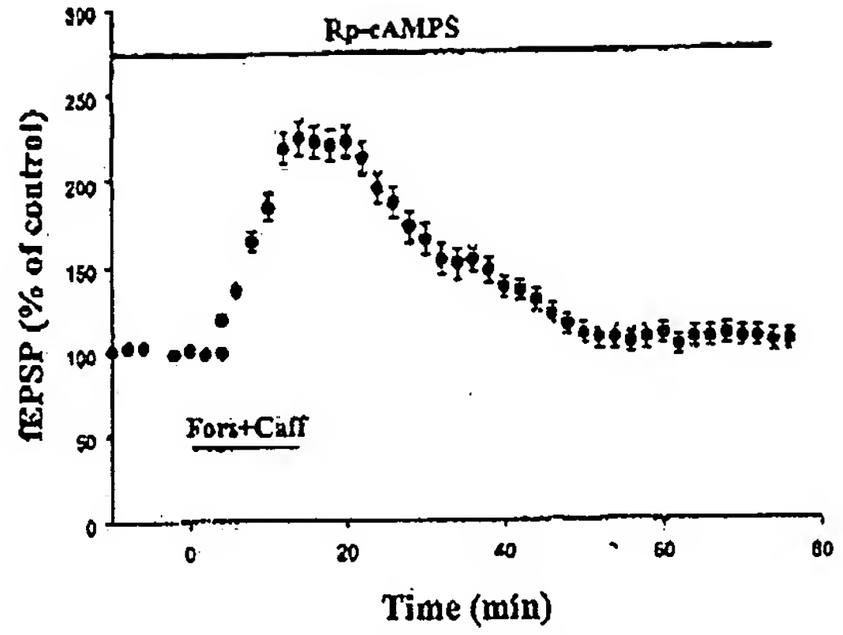

Fig. 6. Promotion of forskolin-induced LTP by caffeine is blocked by Rp-cAMPS. The percent change of fEPSP was plotted as a function of time. Bars denote the application of Rp-cAMPS (25 μM) and forskolin (25 μM)+caffeine (100 μM).

inhibition and adenosine antagonism, we employed enprofylline which has been shown to exhibit differed pharmacological profiles from those of classical methylxanthines owing to its low potency as an adenosine A_1 antagonist (19). Unexpectedly, we found that enprofylline on its own enhanced the fEPSP and shifted the dose-response curve of 2-CA-mediated inhibition to the right. This result indicates that enprofylline does possess adenosine A_1 antagonistic property which increased fEPSP slope by removing tonic inhibition exerted by the endogenous adenosine in this region of the brain. The parallel increase of fEPSP and promotion of LTP observed with enprofylline suggests that adenosine A_1 antagonism is the primary mechanism behind caffeine's promotion of LTP. Consistently, it has been shown that rolipram and Ro20-1724, specific phosphodiesterase inhibitors (3), had no effect on the basal synaptic transmission (2, 17, 21). Finally, this conclusion is further strengthened by the finding that promotion of forskolin-induced LTP is mimicked by the nonxanthine adenosine receptor antagonist CGS 15943. However, we could not rule out the possible involvement of phosphodiesterase inhibitory effect for caffeine to promote LTP because a reagent, which inhibits phosphodiesterase without antagonizing A_1 receptor, was not used in the present study.

Convergent pharmacological and genetic evidence has implicated cAMP and cAMP-dependent protein kinase A (PKA) in the late phase of LTP (L-LTP) in Schaffer collateral-CA1 synapses (1,12,15). In the present study, forskolin at the concentration used (25 μM) did not produce long-term effect on the synaptic transmission (20). Only in the presence of caffeine did forskolin induce LTP suggesting a role played by the adenosine. It is likely that activation of adenylyl cyclase by forskolin resulted in a large

increase in cAMP which left the cell (13, 17, 21, 22) and acted on adenosine receptors to curtail forskolin-induced LTP.

In summary, it is well established that adenosine exerts an inhibitory tone in the mammalian brain, primarily by depressing the release of neurotransmitter (8, 10, 14). Anticipatedly, adenosine antagonists like caffeine enhance transmitter release and exhibit CNS stimulatory, proconvulsant, anxiogenic and antidepressant activities. The results of this study add an additional, long-term effect of caffeine in enhancing the cognitive performance, provided that LTP represents a cellular mechanism for learning and memory (5).

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fatattack

Richard B. Kreider PhD, FACSM

Coleus Forskohlii: One Phat Fat-Fighter!

If you're like me, you're always on the lookout for nutrients and/or herbs that may help reduce body fat without unwanted side effects. In my research, I recently came across an herb called coleus forskohlii that appears to have some promise. Although it has been available for centuries as a medicinal herb in India, the use of coleus forskohlii (containing standardized forskolin) in the U.S. was fairly limited until Twinlab launched it and added coleus forskohlii (standardized for forskolin) to its weight loss supplements (Ripped Fuel Extreme & Ultimate Diet Fuel). So what is this stuff? Can coleus forskohlii really help melt away unwanted fat? This discussion overviews what we know about coleus forskohlii and whether it can help you promote fat loss during training.

What is Coleus Forskohlii?

Coleus forskohlii is a plant that is a member of the mint family. It is most often found growing naturally in the subtropical climates of India, Burma and Thailand. Over the centuries, coleus forskohlii has primarily been used as a food spice and a medicinal herb in the traditional Hindu system of medicine known as Ayurveda.[1,2] According to this natural philosophy of healing, coleus forskohlii may be useful in the treatment of respiratory deficiencies, skin infections and parasitic worms. Although many Eastern herbal remedies have not been extensively studied, a significant amount of research has been conducted over the last 30 years to understand the potential therapeutic benefits of coleus forskohlii. This research has determined that the primary active ingredient in coleus forskohlii is diterpene forskolin, which is primarily found in the root of the plant. Since coleus forskohlii is the only known plant source of forskolin, extracts from coleus forskohlii serve as a natural source of forskolin for nutritional supplements.

What Does Forskolin Do?

Although much of the research on forskolin has been conducted on animals, the basic research findings suggest that it may have several potential health benefits. For example, forskolin has been shown to be a potent non-hormonal activator of adenylate cyclase.[3-5] Adenylate cyclase is an enzyme that activates cyclic adenosine monophospahte (cAMP) in the cell. When activated, cAMP has a number of tissue-specific actions. For example, several studies have indicated that forskolin stimulates cAMP and increases the breakdown of fat (lipolysis) in fat cells.[3-4] In addition, forskolin has been reported to help dilate the bronchial passages and improve respiratory efficiency[6-8]; enhance the ability of the heart to pump blood[9,10]; and lower systemic blood pressure and pressure in the eye.[10,11] Moreover, there is some evidence that forskolin may have anticarcinogenic,[12] anti-inflammatory,[13] and antiathrogenic properties.[5] Theoretically, these findings suggest that forskolin may promote fat loss as well as possess some health and therapeutic benefit. No major side effects have been reported in these studies. →

Does Coleus Forskohlii Promote Fat Loss?

Although the mechanisms of action of forskolin are fairly well known and coleus forskohlii has been available as an herbal supplement for some time, it is presently unclear whether coleus forskohlii supplementation may promote fat loss in healthy individuals. In my research, I was only able to uncover one study that has evaluated the effects of coleus forskohlii on body composition and markers of health.[14] (A second study is underway with Twinlab Ripped Fuel Extreme). In this study, six overweight females underwent medical screening and baseline testing. After this testing, subjects were administered 500 milligrams of a 10 percent forskolin extract (the same amount found in Twinlab Ripped Fuel Extreme and Ultimate Diet Fuel) daily for eight weeks. Subjects ingested one capsule (250mg) in the morning and one in the evening, half an hour before each meal. Subjects were asked to maintain normal activity levels and dietary practices.

Study findings suggest that forskolin may promote fat loss as well as possess some health and therapeutic benefit.

Results revealed that the subjects lost 4.3 and 7.25 pounds of body weight after four and eight weeks of supplementation, respectively. Moreover, bioelectrical impedance (BIA) determined body fat percentage decreased from 33.6 to 25.9 percent after eight weeks of supplementation while lean body mass increased from 67.7 to 74.1 percent of body weight. No significant side effects were reported, although systolic (114 to 105 mmHg) and diastolic (71 to 66 mmHg) blood pressure tended to decrease during the study. While this is not a placebo-controlled double blind study and BIA is not the most accurate method of assessing body composition, these preliminary findings provide some support to contentions that coleus forskohlii may promote fat loss without significant side effects. However, additional well-controlled research is needed before any conclusions can be made.

Bottom Line

Coleus forskohlii (standardized for forskolin) appears to have a number of potentially beneficial physiological effects that may help promote fat loss and/or general health. For this reason, coleus forskohlii has recently been added to several thermogenic weight loss supplements . ■

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Supplement Performance

By Richard B. Kreider PhD, FACSM

Fat Loss Update

Last year I wrote an article about a new herbal supplement that was making waves in the nutrition industry called coleus forskohlii (forskolin). After reviewing the theoretical rationale and available data, I concluded that forskolin appears to have a number of potentially beneficial physiological effects that may help promote fat loss and/or general health. However, additional well-controlled research was necessary to determine the safety and efficacy of forskolin supplementation before definitive conclusions could be drawn. Over the last year, two new studies were conducted on forskolin. This article gives you an inside look at the results of these studies.

Coleus forskohlii is a plant that's a member of the mint family that is grown mainly in India, Burma and Thailand. It has primarily been used as a food spice and to treat respiratory deficiencies, skin infections and parasitic worms in the traditional Hindu system of medicine known as Ayurveda.[1] The primary active ingredient in coleus forskohlii appears to be diterpene forskolin found in the root of the plant. Forskolin has been reported to be a potent non-hormonal activator of adenylate cyclase,[2,4] an enzyme that activates cyclic adenosine monophospahte (cAMP) in the cell. cAMP has a number of metabolic actions including promoting the breakdown of fat (lipolysis) in fat cells.[2,3] In addition, forskolin has also been reported to dilate the bronchial passages and improve respiratory efficiency[6,7]; enhance the ability of the heart to pump blood[8,9]; and lower systemic blood pressure and pressure in the eye.[9,10] There's also some evidence that forskolin may have anti-carcinogenic,[11] anti-inflammatory[12] and antiathrogenic properties.[4] Theoretically, forskolin supplementation may promote fat loss as well as some health benefits.

Recent research suggests that supplementing the diet with a thermogenic supplement containing coleus forskohlii can promote fat loss without the typical loss of muscle mass observed in weight loss studies.

Until recently, only one pilot study evaluated the effects of coleus forskohlii on body composition and markers of health in humans.[13] In this study, six overweight females underwent medical screening and baseline testing. Then the subjects were administered 250 milligrams of a 10 percent forskolin extract (Forslean®, Sabinsa Corp., Piscataway, NJ) twice daily for eight weeks. Subjects were asked to maintain normal activity levels and dietary practices during the study. Results revealed that the subjects lost 4.3 and 7.25 pounds of body weight after four and eight weeks, respectively. Moreover, bioelectrical impedance (BIA)- determined body fat decreased from 33.6 to 25.9 percent, while lean body mass increased from 67.7 to 74.1 percent of body weight after eight weeks. No clinically significant side effects were reported, although systolic (114 to 105 mmHg) and diastolic (71 to 66 mmHg) blood pressure tended to decrease. Although this study was not well controlled (i.e., double blind, placebo controlled, randomized), these preliminary findings provided some support to contentions that coleus forskohlii supplementation may promote fat loss without significant side effects.

Over the last year, two studies have evaluated the effects of ingesting supplements containing coleus forskohlii on body composition and markers of medical safety. In the first, Dr. Carlon Colker and colleagues[14] evaluated the effects of supplementing the diet with

catch a fish and win half a million bucks. No disrespect to those athletes. I couldn't do those things, yet I find them intriguing.

The point is, our sport can be interesting. There's tons of beautiful girls and guys, free prizes, meet-and-greets, awesome nightlife on the Vegas strip, plenty of partying, and the cool thing is, everyone there is into it! When the lights fade to black, and the bodybuilding show is on, we see some cartoon looking gladiators stalking the stage, posing, doing their routines, and trying to win the crown. Our sport isn't for everyone, but you cannot tell me watching real life gladiators— *gladiators*, dawg— who look like they are from another planet with their game face ready to do battle is any more boring than golf, skateboarding, or fishing. These sports all have huge corporations behind them to the point where the athlete placing last goes home with more money than the winner at the Olympia. The problem is the organization isn't making a concerted effort to let the general public know why this sport is cool and why they should watch it! Its strange, but strange sells.

MD: How big is your dick?

FW: [Stutters] What!?

MD: [Laughs] Sorry, I wanted to change the pace! [More laughter]

FW: Rico is right. You need help!

Rico: [Disgusted, shakes head] Told ya.

MD: Forget I said it. Anyway, did you have a good time at the Olympia?

FW: Yeah, I sure did. I was nervous about going. I waited until the last minute, but was greeted by everyone with open arms about my decision. That touched me deeply. Even the athletes I used to do battle with were very kind and understanding, you know? I would like to take this opportunity to address the athletes, including Shawn Ray. I want everyone to know that what I've said here is just my opinion, and hope no one takes it personally. If they do, I pray for their forgiveness. Any past rivalries, bitterness and conflicts, I wish to squash them right here and now. I'm sorry to those who I may have affected negatively when I was not myself back in the day. These athletes are superstars and should be treated as such. The readers deserve more. Let's all end the ego-driven negativity, and do what's best for the sport and everyone involved from top to bottom. Long live bodybuilding; the sport that has given myself and thousands of others so much.

Right now, our world is concerned over much more substantial things. Anthrax is going around, another plane just crashed in New York, and we are fighting a critical war with serious religious overtones. In the grand scheme of things, bodybuilding politics mean so little, but the activity itself is so rewarding I rate it on par with martial arts— a discipline. Going to the gym, releasing tension, pushing yourself physically, changing your life for the better, and daring to dream is the essence of bodybuilding. Let's focus more on fixing the sport, because it needs change and this can easily be done. The ball is in our court to go for the slam dunk of success, but the question is, will the major players give us the lay-up to make it happen.

MD: Well said. We'll end here. Thanks for the interview, Flex. And Rico. [Smiles]

FW: Ahh, I'm glad its over! [Laughs]

Rico: The door is that way, Tyrell.

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Ripped Fuel Extreme™ on body composition and markers of health. This supplement contains MaHuang (standardized for 20mg of ephedra alkaloids per serving), guarana (standardized for 200mg of caffeine per serving), citrus aurantum (standardized for 5 mg of synephrine alkaloids), green tea leaf extract (standardized for polyphenols), Cycloburn™ coleus forskohlii root extract (standardized for 16.7 mg of forskolin) and other nutrients.

The researchers recruited 26 overweight subjects to participate in this study. Subjects were divided into two groups and randomly assigned to ingest in a double blind manner either a placebo or Ripped Fuel Extreme. Each group ingested one capsule three times per day for seven days and then two capsules with each of three daily meals. During the study, subjects maintained a slightly hypocaloric diet (25 kcal per kilogram of body weight per day) and participated in a monitored exercise program performed three times per week.

Results revealed that there were no changes in heart rate, blood pressure, oxygen saturation, resting electrocardiograms (ECG), pulmonary function, or a standard panel of markers of clinical safety in the blood during the course of the study for either group. Subjects who supplemented their diets with Ripped Fuel Extreme lost 5.6 pounds and 2.1 percent of their BIA-determined body fat while subjects in the control group only lost about one pound. Interestingly, Dr. Colker reported that despite this weight loss, fat-free mass was maintained, which is somewhat unusual in these types of studies. While the specific impact that forskolin had on these results is unclear, these findings add to the growing body of evidence that responsible use of thermogenic supplements containing ephedra alkaloids and caffeine can effectively promote weight loss while not adversely affecting health status in apparently healthy individuals.


PER BERNAL

Researchers in my lab recently conducted a pilot study to examine the effects of coleus forskohlii supplementation on weight loss in sedentary overweight females.[15] In this study, 23 moderately overweight females were administered in a double blind and randomized manner a capsule containing a placebo or Forslean™ (standardized for 25 mg of forskolin) two times per day for 12 weeks. Body mass, total body water (BIA), body composition (dual energy x-ray absorptiometry [DEXA]) and psychometric instruments were obtained at zero, four, eight and 12 weeks of supplementation. Fasting blood samples and dietary records were obtained at weeks zero and 12. In addition, side effects were monitored by a research nurse and recorded on a weekly basis.

Results revealed no significant differences in caloric or macronutrient intake between groups. Subjects who supplemented their diets with forskolin lost a little body weight (-1.5 pounds) while the subjects that received the placebo gained weight (+2.2 pounds). No significant differences were observed in changes in fat mass, fat-free mass, or percent body fat. However, subjects who took forskolin tended to feel less fatigue and hunger, and more of a feeling of fullness. No clinically significant interactions were observed in metabolic markers, blood lipids, muscle and liver enzymes, electrolytes, red cells, white cells, insulin, or thyroid hormones. In addition, no significant differences were observed in heart rate, blood pressure, or weekly reports of side effects. Although more research is needed, these findings suggest that Forslean may help mitigate weight gain in overweight females with apparently no clinically significant side effects.

Bottom Line

Recent research suggests that supplementing the diet with a thermogenic supplement containing coleus forskohlii can promote weight loss without the typical loss of muscle mass observed in weight loss studies. In addition, supplementing the diet with coleus forskohlii may help overweight women prone to gaining weight slightly

decrease body mass and affect some psychological perceptions of fatigue and hunger. In both studies, the supplements appeared to be well tolerated. These findings provide additional support to contentions that forskolin may modestly affect body composition. However, additional well-controlled research is necessary to determine the safety and efficacy of coleus forskohlii supplementation before definitive conclusions can be drawn. ☐

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potentiate the actions of forskolin.

Effects of Coleus Forskohlii Supplementation on Body Compostion and Markers of Health in Sedentary Overweight Females.
FASEB J. 2002; 16 Suppl: 59. (LB305)
Kreider R, Henderson S, Magu B, Rasmussen C, Lancaster S, Kerksick C, Smith P, Melton C, Cowan P, Greenwood M, Earnest C, Almada A.

In a double blind and randomized manner, 23 females supplemented their diet with *Coleus Forskohlii* (250 mg of ForsLean® with 10% forskolin, n=7) or a placebo (n=12) two times per day for 12-wks. Body composition (DEXA), body weight, and psychometric instruments were obtained at 0, 4, 8, and 12 weeks of supplementation. Fasting blood samples and dietary records (4-d) were obtained at 0 and 12-wks. Side effects were recorded on a weekly basis. Data were analyzed by repeated measures ANOVA and are presented as mean changes from baseline for the CF and placebo groups, respectively. No significant differences were observed in caloric or macronutrient intake. CF tended to mitigate gains in body mass (-0.7±1.8, 1.0±2.5 kg, p=0.10) and scanned mass (-0.2±1.3, 1.7±2.9 kg, p=0.08) with no significant differences in fat mass (-0.2±0.7, 1.1±2.3 kg, p=0.16), fat free mass (-0.1±1.3, 0.6±1.2 kg, p=0.21), or body fat (-0.2±1.0, 0.4±1.4% p=0.40). Subjects in the CF group tended to report less fatigue (p=0.07), hunger (p=0.02), and fullness (p=0.04). No clinically significant interactions were seen in metabolic markers, blood lipids, muscle and liver enzymes, electrolytes, red cells, white cells, hormones (insulin, TSH, T3, and T4), heart rate, blood pressure, or weekly reports of side effects. Results suggest that CF may help mitigate weight gain in overweight females with apparently no clinically significant side effects.

Related Products: Acti-Cyclase

thematic review

BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis

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Abstract During the last three years there have been a plethora of publications on the liver X-activated receptors (LXRα, NR1H3, and LXRβ, NR1H2), the farnesoid X-activated receptor (FXR, NR1H4), and the pregnane X receptor (PXR, NR1I2) and the role these nuclear receptors play in controlling cholesterol, bile acid, lipoprotein and drug metabolism. The current interest in these nuclear receptors is high, in part, because they appear to be promising therapeutic targets for new drugs that have the potential to control lipid homeostasis. In this review we emphasize *i*) the role of LXR in controlling many aspects of cholesterol and fatty acid metabolism, *ii*) the expanded role of FXR in regulating genes that control not only bile acid metabolism but also lipoprotein metabolism, and *iii*) the regulation of bile acid transport/metabolism in response to bile acid-activated PXR.— Edwards, P. A., H. R. Kast, and A. M. Anisfeld. BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis. *J. Lipid Res.* 2002. 43: 2–12.

Supplementary key words bile acids • oxysterols • Tangier disease • sitosterolemia • ABC transporters • lipoproteins • farnesoid X-activated receptor • liver X-activated receptor • pregnane X receptor

INTRODUCTION

1. Nuclear receptors, hormone response elements, and activated transcription

Nuclear receptors, which include both non-steroidal and steroid receptors, bind to DNA *cis* elements, known as hormone response elements (HREs) and activate transcription of target genes. Most non-steroidal receptors, such as LXR, FXR, and PXR (liver X-activated receptor, farnesoid X-activated receptor, and pregnane X receptor, respectively), bind DNA as heterodimers with the obligate partner 9-*cis* retinoic acid receptor α (RXRα, NR2B1) (1).

HREs are usually composed of direct, inverted, or everted repeats (DR, IR, or ER, respectively) of the idealized sequence AGGTCA that are separated by a variable number (n) of nucleotides (where n = 1–6) to give DRn, IRn, or ERn hormone response elements (2). In general, each nuclear receptor preferentially binds to a limited number of HREs that have a particular spacing and orientation, thus providing specificity for the formation of the protein-DNA complex (2). Functional HREs have been identified in the proximal promoters of target genes, in distal enhancers that can be located ≥22 kb from the transcription start site and in introns (see below).

LXR, FXR, PXR, and RXRα, like other members of this large superfamily of transcription factors, have a number of specific functional domains that usually include a poorly understood amino terminal transcriptional activation domain (AF-1), a DNA binding domain (DBD), a ligand binding domain (LBD), domains responsible for nuclear translocation and dimerization, and a transcriptional activation domain (AF-2) at the extreme carboxyl terminus (Fig. 1) (1, 3). In general, transcriptional activation is dependent on the entry of a specific ligand, usually a small lipophyllic molecule, into the cavity formed by the LBD of the nuclear receptor. Many, but not all, non-steroidal nuclear receptors are thought to be pre-bound to the HRE in a complex with corepressor proteins. Entry of the ligand into the LBD initiates changes in the conformation of the receptor that results in loss of corepressor proteins, recruitment of coactivator proteins and increased transcription (4). The role of the corepressor and coactivator proteins in controlling the condensed state of the DNA, via acetylation and deacetylation, has been reviewed recently, but is beyond the scope of the current article (4, 5).

2. Orphan nuclear receptors

Like many other nuclear receptors, LXR, FXR, and PXR were originally termed orphan nuclear receptors because their natural ligands were unknown at the time that they were initially cloned. With the recent identification of several physiological ligands that activate LXR, FXR, or PXR we can consider that these orphans have been "adopted". These

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Abbreviations: apoC-II, apolipoprotein C-II; BARE, bile acid response element; FXR, farnesoid X-activated receptor; HRE, hormone response element; LXR, liver X-activated receptor; PXR, pregnane X receptor; RXR, 9-*cis* retinoic acid receptor; SREBP, sterol response element binding protein.

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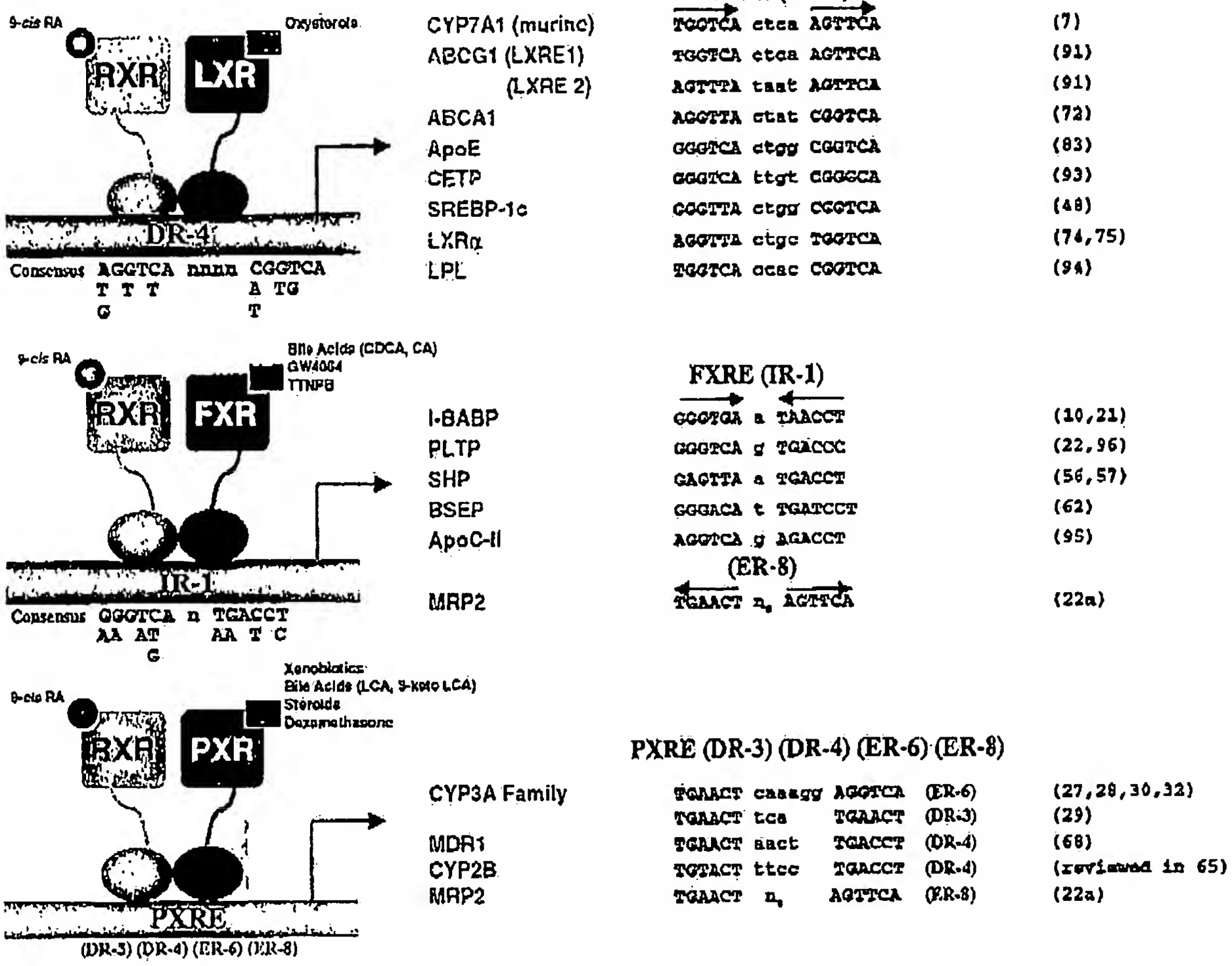

Fig. 1. LXR, FXR, and PXR (liver X-activated receptor, farnesoid X-activated receptor, and pregnane X receptor, respectively) target genes and their hormone response elements. The ligands that activate the indicated nuclear receptor heterodimers are shown in the cartoon on the left. The known target genes are shown opposite the corresponding heterodimer together with the nucleotide sequence of the hormone response element (LXRE, FXRE, PXRE). The consensus sequence for the LXRE (DR-4) and FXRE (IR-1) are shown on the left. The color of the target genes for LXR (green), FXR (red), and PXR (blue) are conserved in Figs. 1–3.

adoptions resulted from a series of elegant studies that identified *i*) oxysterols [e.g., 24(*S*),25-epoxycholesterol, 20(*S*)-, 22(*R*)-, 24(*S*)-, and 27-hydroxycholesterol] as activators of LXR (6–8), *ii*) primary bile acids, such as chenodeoxycholic acid (CDCA) and cholic acid (CA), as activators of FXR (9–11), and *iii*) the secondary bile acid, lithocholic acid (LCA) and its metabolite 3-keto LCA, as activators of PXR (12, 13) (Fig. 1). The identification of these natural ligands and the generation of mice with deletions in the genes encoding LXRα, LXRβ, FXR, and PXR have opened up new vistas linking the role of these receptors to regulatory functions.

3. Oxysterol- and bile acid-activated nuclear receptors (LXR, FXR, and PXR)

A. LXR. LXR was originally isolated from a human liver cDNA library and shown to be most highly expressed in this tissue (14). Subsequently, two genes were identified, LXRα and LXRβ (also called RLD-1 and OR-1, respectively), that encode highly conserved isoforms. LXRα is expressed in a tissue specific manner, whereas LXRβ is ubiquitously expressed (15). Both isoforms bind DNA as a heterodimer with the common partner RXR. LXR/RXR binds preferentially to hormone response elements (LXREs) that consist of two idealized hexanucleotide repeats (AGGTCA) separated by four nucleotides (DR-4) (Fig. 1) (14). Subsequently, screens were developed that led to the identification of specific oxysterols as activating ligands for LXR (6–8). The most potent oxysterols included 24(*S*),25-epoxycholesterol, 22(*R*)-hydroxycholesterol, and 24(*S*)-hydroxycholesterol (7, 8, 16).

Based on studies with LXR null mice and the identification of a limited number of LXR target genes (Fig. 1), it appears that LXR functions as a sensor of cellular oxysterols. Consistent with this proposal, all LXR target genes encode proteins that have major roles in controlling cholesterol and/or fatty acid homeostasis in a number of tis-

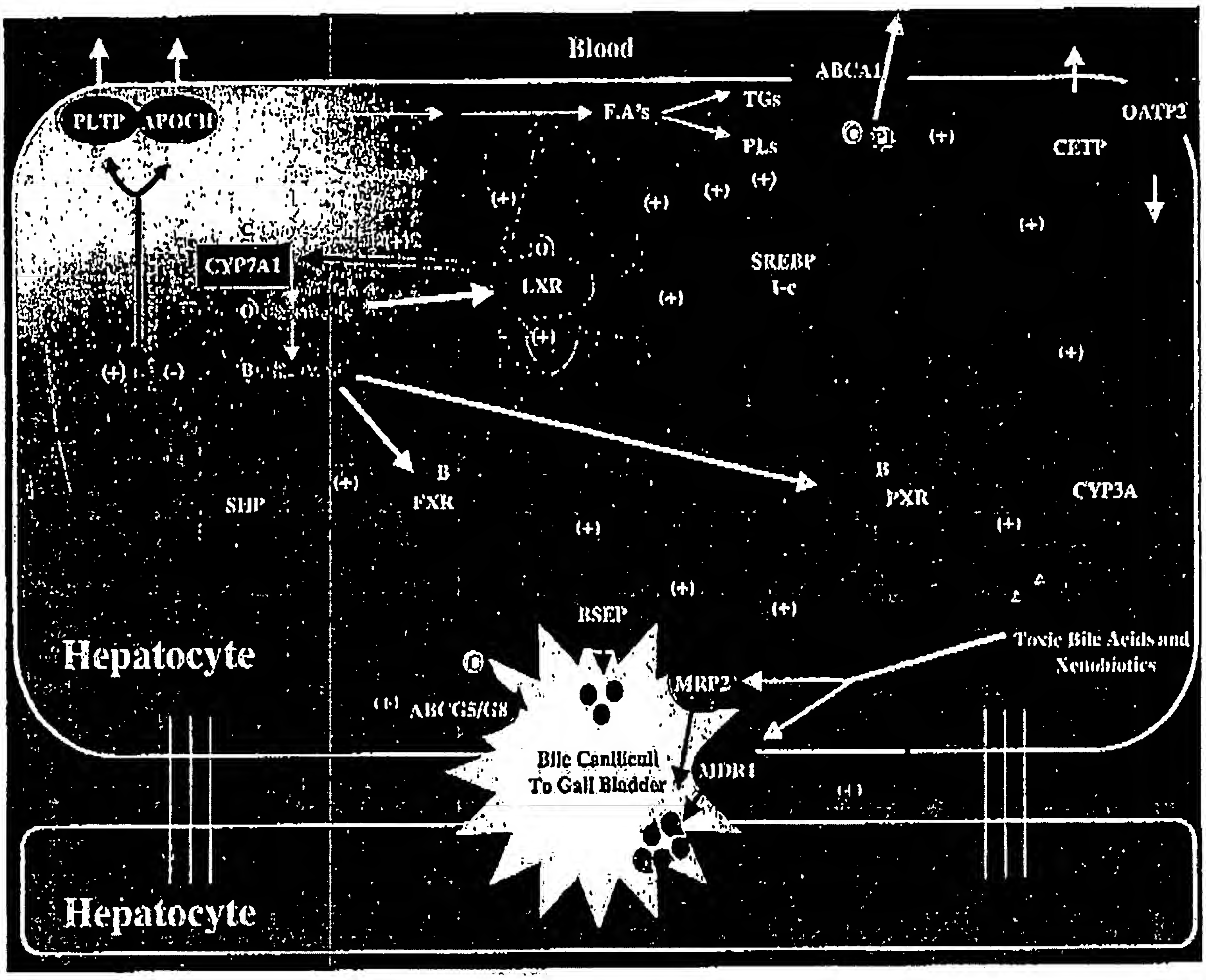

Fig. 2. LXR, FXR, and PXR regulate the hepatic expression of genes involved in lipid homeostasis. The conversion of acetyl-CoA to cholesterol or fatty acids (FA), phospholipids (PL), or triglycerides (TG) is indicated. The nuclear receptors LXR (green), FXR (red), and PXR (blue) are color-coded the same as their target genes. Transcriptional activation (+) or repression (−) is indicated. Bile acids (B), cholesterol (C), and oxysterols (O) are drawn as colored spheres. The cellular locations of ABCG1, ABCG5, and ABCG8 have not been established. FPP, farnesyl diphosphate. All other abbreviations are indicated in the text.

sues including the liver, intestine, macrophages and possibly adipose tissue (Fig. 1, Fig. 2, and Fig. 3). However, the relative importance of LXRα and LXRβ as sterol sensors and their specific roles in regulating gene expression is poorly understood. Recently, synthetic, highly potent LXR ligands were shown to have pronounced effects in vivo. For example, treatment of rodents with such agonists resulted in decreased cholesterol absorption and increased concentrations of plasma triglycerides and phospholipids (17).

B. FXR. Rat FXR was originally cloned using PCR and degenerate primers corresponding to the semi-conserved DNA binding domain of nuclear receptors (18). At the same time, murine FXR was isolated based on its interaction with RXR and, as a result, was originally referred to as RIP-14 (RXR interacting protein number 14) (19). In the original report, rat FXR was shown to be weakly activated by supraphysiological levels of the isoprenoid farnesol (hence the name FXR) (18). Farnesol is derived from the hydrolysis of farnesyl diphosphate in the isoprenoid biosynthetic pathway (Fig. 2) and had previously been shown to function as a signaling molecule in an unrelated pathway that controls the stability of HMG-CoA reductase (20). However, no direct interaction of farnesol with FXR or the LBD of FXR was ever demonstrated. More recent studies have shown that primary bile acids, such as CDCA or CA, bind to FXR in vitro, that this interaction occurs at physiological levels of the bile acids (EC_{50} of 10–15 μM), that this interaction results in recruitment of coactivators to the liganded FXR, and that there is a subsequent increase in the transcription of target genes (9–11). All of these properties are consistent with the hypothesis that CDCA and CA function to directly activate FXR in vivo.

The hormone response element to which FXR/RXRα binds was originally termed an FXR response element (FXRE) (18). This element, however, has also been termed a BARE (bile acid response element), based in part on the more recent studies which indicate that bile acids are the natural ligands for FXR (10, 21). Earlier re-

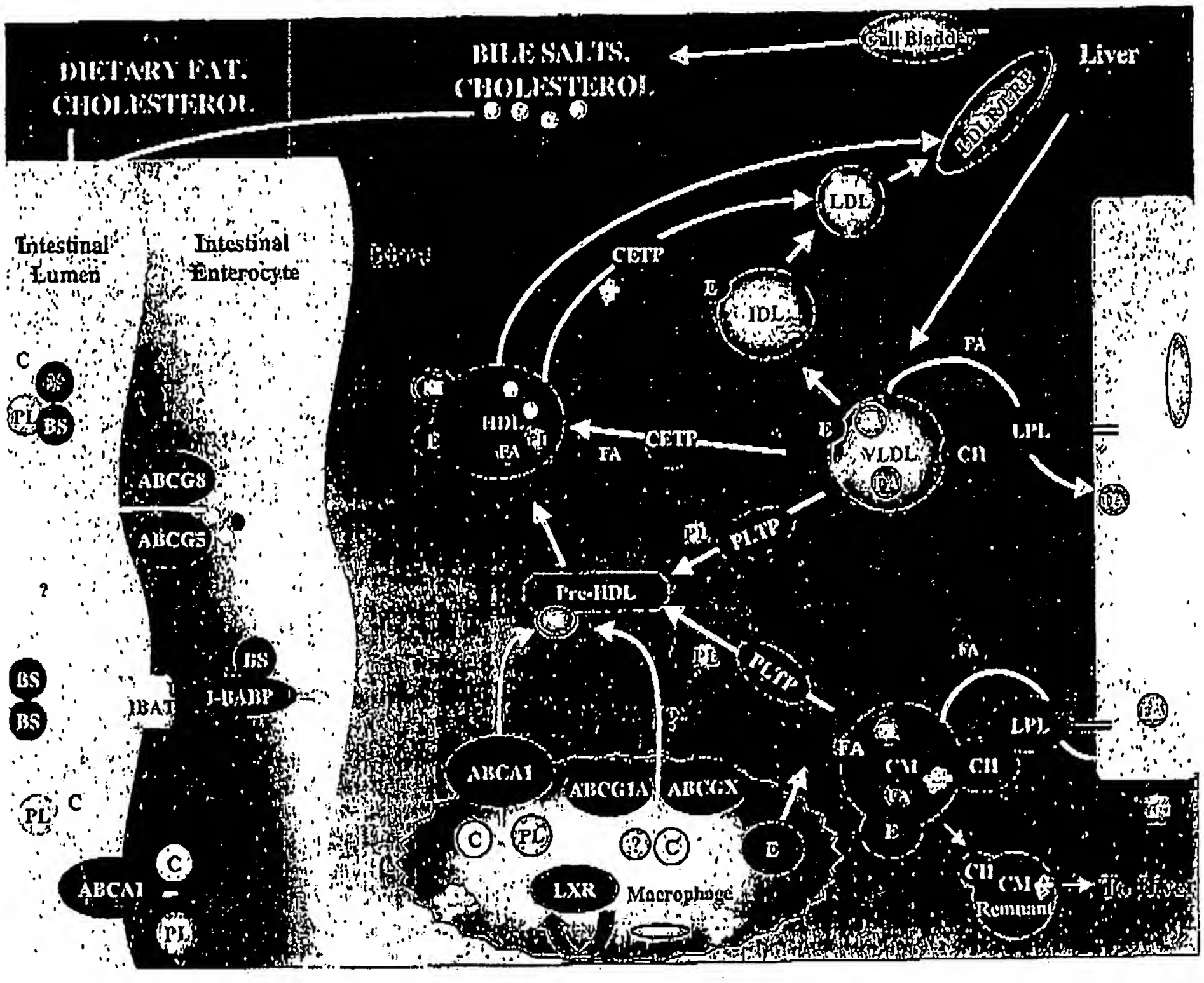

Fig. 3. LXR and FXR regulate genes involved in lipid absorption, excretion, and metabolism. The figure illustrates lipid absorption from the intestinal lumen into the villi, the metabolism of lipoproteins in the plasma and the movement of lipids out of macrophages or the liver. Genes that are activated by LXR are shown in green, while FXR target genes are shown in red. The membrane localization of ABCG1A, ABCGX, ABCG5, and ABCG8 are unknown. Other details are provided in the text. The following abbreviations are used: cholesterol (C, yellow droplets; FA, fatty acids; PL, phospholipids; BS, bile salts; IBAT, ileal bile acid transporter; I-BABP, ileal bile acid binding protein; AI, apolipoprotein A-I; E, apolipoprotein E; C-II, apolipoprotein C-II; PLTP, phospholipid transfer protein; LPL, lipoprotein lipase; CM, chylomicrons; CM remnant, chylomicron remnant; LDLR, LDL receptor; ABCG1A, ABCGX, represent different ABCG1 isoforms).

ports show that various degenerate forms of IR-1, DR-3, or DR-4 elements were bound by FXR/RXRα in vitro (18, 22). With one exception, all published FXR target genes contain one or more degenerate IR-1 elements in the proximal promoter or distal enhancers that function as an FXRE/BARE and are required for transcriptional activation (Fig. 1). In recent studies, we identified an ER-8 as a functional FXRE/BARE that is distinct from all previously identified nuclear hormone response elements (22a). Such an observation indicates that the nucleotide sequence of a functional FXRE/BARE may vary considerably.

Northern blot assays indicate that FXR transcripts in the rat are restricted to the liver, kidney, intestine (all involved in cholesterol/bile acid metabolism), colon, and adrenals (18). As discussed below, the original proposal that FXR might have a role in cholesterol/bile acid homeostasis has proven to be correct (Fig. 2) (18). However, the role of FXR in the adrenals is an enigma, since this organ is not known to be involved in any aspect of bile acid metabolism. The finding that androsterone, an intermediate in cholesterol/steroid metabolism, is both synthesized in the adrenals and induces the expression of a reporter gene under the control of an FXRE is certainly intriguing (23). Demonstration that androsterone, at physiological concentrations, can both bind FXR and activate endogenous FXR-target genes would provide further support for the proposal that this steroid is a natural agonist.

C. PXR. The secondary bile acid, lithocholic acid (LCA), is produced from the primary bile acid CDCA by a 7α-dehydroxylation pathway present in certain intestinal bacteria. In contrast to primary bile acids, secondary bile acids are poorly absorbed in the distal ileum. Since LCA has recently been shown to function as a ligand for PXR (see below), we have included a brief section on this nu-

clear receptor. Far more extensive reviews on PXR are available (24–26).

Murine PXR and its human homolog (hPXR) (also called the steroid and xenobiotic receptor, SXR, or the pregnane activated receptor, PAR) were first cloned in 1998 (27–30). In addition to LCA, a number of naturally occurring steroids, including pregnenolone, progesterone, androstanol, hyperforin (a component of St John's Wort), dexamethasone (a synthetic glucocorticoid), and various xenobiotics (e.g., rifampicin and phenobarbital) have been shown to activate PXR (Fig. 1) (29–32). The observation that a number of compounds differentially activate murine and human PXR is noteworthy as this may explain the varying responses of mice and humans to certain drugs.

The recent observations that the secondary bile acid LCA and its 3-keto metabolite (3-keto LCA) activate PXR are particularly relevant to this review. Accumulation of these natural compounds in the liver is associated with toxicity and cholestasis (12, 13). As discussed in detail below, the data suggest that PXR functions as a hepatic sensor for many xenobiotics, natural steroids and certain bile acids. Activated PXR then induces the expression of genes that are involved in hepatic uptake, metabolism and subsequent excretion of many of the same compounds (Figs. 1, 2).

THE ROLE OF LXR, FXR, AND PXR IN CELLULAR CHOLESTEROL AND BILE ACID METABOLISM

1. Intestine

A. Role of LXR in the intestine. Three genes (ABCA1, ABCG5, and ABCG8) that encode ATP-binding cassette (ABC) transporter proteins have been proposed as LXR target genes in the intestine (33–37). ABCG5 and ABCG8 appear to function by limiting the intestinal absorption of sterols (cholesterol and/or plant sterols) and enhancing the excretion of sterols from the liver into the bile (33, 35, 38). In contrast, ABCA1 appears to both facilitate the efflux of phospholipids and cholesterol from a variety of cells and also to limit cholesterol absorption in the intestine. As discussed below, these conclusions are based on the phenotype of patients or mice with mutations in these genes as well as the effects of activation of these genes by LXR agonists.

Patients with mutations in the ABCA1 gene have Tangier disease. These patients have little or no plasma HDL (35, 39–41). As expected, deletion of the ABCA1 gene in mice also results in a significant reduction in plasma HDL concentrations (42–44). However, it is perplexing that deletion of the ABCA1 gene in mice is reported to either decrease (45) or increase (42) the rate of absorption of cholesterol from the diet. Consistent with the latter study, it was recently reported that administration of a synthetic LXR agonist to mice resulted in increased intestinal expression of ABCA1 and a concomitant decrease in cholesterol absorption in wild-type but not LXRαβ double knockout mice (37). Based on these studies it has been proposed that ABCA1 may reduce cholesterol absorption by facilitating the efflux of cholesterol from the enterocyte back into the lumen. However, other LXR target genes, in addition to ABCA1, are also induced following the treatment of rodents with LXR agonists. Thus, we cannot rule out the possibility that these other LXR target genes have a role in decreasing sterol absorption. In addition, recent studies with cultured cells indicate that the primary role of ABCA1 is to promote phospholipid efflux to exogenous lipid-poor protein acceptors, such as apoA-I, and that the lipid/protein complex then functions as a sink to receive cellular cholesterol (see below). It remains to be established whether ABCA1 expressed in enterocytes also alters phospholipid movement and thus modulates cholesterol absorption.

Mutations in ABCG5 or ABCG8 result in sitosterolemia (33, 34). Patients with sitosterolemia have elevated levels of cholesterol and plant sterols, especially sitosterol, in both blood and tissues, and show evidence of premature coronary atherosclerosis (33, 34, 38). These increased levels appear to result from hyper absorption of sterols (cholesterol and plant sterols) and a defect in the excretion of plant sterols from the liver into bile (Fig. 3). Since patients with sitosterolemia have mutations in either ABCG5 or ABCG8, it seems likely that these transporters form a functional heterodimer (33). Identification of both the cellular location of these proteins and the substrate that they transport would provide critical information that is currently lacking.

It has been suggested that ABCA1 and ABCG5/ABCG8 function to pump phospholipids and/or sterols out of intestinal enterocytes, hepatocytes and/or macrophages (Figs. 2, 3). Since these three genes are all activated by LXR there is considerable clinical interest in determining whether treatment with LXR agonists will be beneficial as a result of either decreasing cholesterol absorption or increasing the efflux of lipids from macrophage foam cells in the artery wall.

B. Role of FXR in the intestine. The first gene to be identified as a direct target of ligand-activated FXR was I-BABP (ileal bile-acid binding protein) (10). An IR-1 was identified in the proximal promoter and shown to function in transcriptional activation of the I-BABP gene in response to FXR and bile acids (10, 21). I-BABP is a soluble protein that is expressed in enterocytes and binds bile acids. Consequently, I-BABP may limit the free concentration of bile acids intracellularly and thus limit bile acid-induced toxicity (Fig. 3). The identification of I-BABP as an FXR target gene is consistent with the proposal that FXR plays a central role in regulating bile acid metabolism.

2. Liver

A. Role of LXR in the liver. Hepatic LXR target genes identified to date function to control bile acid synthesis, and metabolism, cholesterol movement, fatty acid synthesis, and lipoprotein metabolism (Figs. 1–3). The availability of mice in which one or both LXR gene(s) have been deleted (LXRα$^{-/-}$, LXRβ$^{-/-}$, or LXRα/β$^{-/-}$) has been particularly useful in understanding the function of these two nuclear receptors. The results of a number of studies

demonstrate that LXRα is necessary for mice to respond appropriately following the administration of cholesterol-enriched diets (46). One important target is the gene encoding cholesterol 7α-hydroxylase.

The hepatic enzyme cholesterol 7α-hydroxylase catalyses the rate limiting reaction in the conversion of cholesterol to bile acids in the classic (neutral) pathway, and consequently plays a critical role in cholesterol and bile acid homeostasis (47). This enzyme is encoded by the highly regulated CYP7A1 gene (47). An alternative pathway has been described that involves the hydroxylation of oxysterols by oxysterol 7α-hydroxylase (CYP7B) prior to their conversion to primary bile acids (47). Recent studies have shown that the rodent CYP7A1 gene is activated by LXR and oxysterols by a process that depends on a functional LXRE in the proximal promoter of the gene (46) (Figs.1. 2). Consistent with these observations, administration of a diet supplemented with high levels of cholesterol increases the expression of CYP7A1, bile acid synthesis and excretion in wild-type mice, but not in LXRα$^{-/-}$ or LXRα/β$^{-/-}$ mice (46, 48, 49). As a result, LXRα/β$^{-/-}$ and LXRα$^{-/-}$ mice, but not wild-type or LXRβ$^{-/-}$ mice, accumulate cholesterol to toxic levels in the liver (46, 49). The physiologically important oxysterol(s) that function as LXR activators and are presumably generated from dietary cholesterol, remain to be identified. Prime candidates include 24(*S*),25-epoxycholesterol, 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol and 27-hydroxycholesterol. The proximal promoter of the human CYP7A1 gene does not contain an LXRE and consequenrly it is not activated by oxysterols or LXR. Thus, the regulation of the murine and human genes are not identical.

Other LXR target genes that are induced in the liver include ABCA1. ABCG5, and ABCG8. Additional studies should help to determine whether these ABC transporter proteins facilitate the efflux of specific phospholipids, cholesterol and/or plant sterols from the liver into either the blood or the bile.

In the last few months it has become apparent that LXR plays a critical role in activating genes involved in fatty acid synthesis (Figs. 1, 2). Presumably, the increased levels of fatty acids are then made available for esterification of excess cholesterol and for the synthesis of triglycerides and phospholipids. In this regard, it has long been known that excess cellular cholesterol is rapidly esterified with an unsaturated fatty acid to form cytoplasmic cholesteryl ester lipid droplets and that this reaction is catalyzed by the membrane-bound enzyme acyl-CoA:cholesterol acyltransferase (ACAT) (50). Interestingly, LXR has recently been shown to activate the sterol regulatory element-binding protein 1c (SREBP-1c) gene, which encodes a transcription factor that is itself critical for the increased expression of a number of genes involved in the biosynthesis and esterification of unsaturated fatty acids (Figs. 1, 2) (51, 52). Such SREBP-1c-activated genes include acetyl-CoA synthetase. acetyl-CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase, glycerol phosphate acyltransferase and CTP:phosphocholine cytidylyltransferase (Fig. 2) (53–55). Surprisingly, oxysterols selectively suppress the nuclear levels of SREBP-2, as compared to SREBP-1 (51, 52). As a result, SREBP-2-dependent genes (e.g., LDL receptor, HMG-CoA reductase) are repressed by oxysterols whereas hepatic SREBP-1c-dependent genes are activated.

B. Role of FXR in the liver. FXR is highly expressed in the liver where it appears to function as a bile acid sensor. Earlier studies had shown that administration of bile acids to rodents repressed the expression of hepatic CYP7A1. Subsequent studies demonstrated that the repression of CYP7A1 was indirect and involved two other nuclear hormone receptors named SHP (NR0B2) and LRH-1 (NR5A2). It was shown that *i*) the gene encoding SHP was activated directly by FXR (an FXRE/BARE was identified in the proximal promoter of the SHP gene), *ii*) there was an increase in SHP protein. and *iii*) the SHP protein formed a complex with, and inactivated, the transcription factor LRH1/CPF (56. 57). Since LRH1 is required for transcription of CYP7A1 (58), one net result of activating FXR and SHP expression was a decrease in the mRNA, protein and activity of CYP7A1 (Figs. 1, 2). Bile acids also repress the synthesis of cholic acid by repressing the transcription of CYP8B1 (sterol 12α-hydroxylase) via a similar mechanism that also involves the inactivation of LRH1 by SHP (59). Thus, bile acids function as end product inhibitors and repress the synthesis of both chenodeoxycholic acid and cholic acid as a result of the decreased expression of CYP7A1 and CYP12B1, respectively (Fig. 2). Alternative mechanisms of regulation of CYP7A1 have also been reported that involve the bile acid-dependent induction of cytokines from Kupffer cells and the subsequent cytokine-mediated suppression of CYP7A1 in adjacent hepatocytes (60). Proof of this model will require studies with SHP null mice.

Studies with FXR null mice provided additional data that support the proposal that FXR is an important sensor of bile acids (61). For example, FXR null mice exhibited severe wasting or death following the inclusion of cholic acid in the diet (61). One putative FXR target gene identified in the study with FXR null mice encoded BSEP (bile salt export pump), which has recently been characterized as a true FXR target (62). BSEP (ABCB11) is a member of the ABC superfamily of transporters that is located on the canalicular membrane of hepatocytes where it facilitates the transport of bile acids from the hepatocyte into the bile (Fig. 2) (63, 64). Thus, identification of FXR target genes is consistent with an important role of this nuclear receptor in regulating *i*) bile acid synthesis in the liver (via SHP), *ii*) excretion of bile acids into the bile (BSEP), and *iii*) the re-uptake of bile acids from the intestinal lumen (I-BABP) (Figs. 2, 3).

C. Role of PXR in the liver. Recent studies have demonstrated that mRNA levels of the murine sodium-independent organic anion transporting polypeptide (OATP2) and specific cytochrome P450 (CYP) genes such as CYP3A and CYP2B are induced when hepatocytes are incubated with LCA, 3-keto LCA, or a number of other PXR ligands (Figs. 1, 2) (12, 13). CYP genes encode enzymes involved in hydroxylation and metabolism/inactivation of numerous drugs, xenobiotics, and some bile acids (65). Rodent OATP2 functions to transport organic anions and sulfated

and glucuronidated bile acids from the blood into hepatocytes, whereupon these compounds may be hydroxylated by CYP3A prior to their excretion in the bile (Fig. 2) (66, 67). Interestingly, the excretion of many of these compounds into the bile occurs via the transmembrane transporter MDR1 (ABCB1) located on the cannalicular membrane (Fig. 2) (68). Since MDR1 is also induced by PXR, it appears that this nuclear receptor activates genes involved in the hepatic uptake of anions from the blood, their hydroxylation and their subsequent excretion.

Activated PXR, like activated FXR, results in repression of CYP7A1 (12). However, the mechanism of this repression remains to be determined. Nonetheless, CYP7A1 appears to be a central target for multiple nuclear receptors; CYP7A1 expression is repressed by both bile acid-activated PXR and FXR, and induced by oxysterol-activated LXR. Such data suggest that in mammals, the rate of conversion of cholesterol to bile acids is a particularly important process.

Studies with $PXR^{+/+}$ mice and $PXR^{-/-}$ mice that overexpress constitutively activated human PXR ($PXR^{-/-}$ SXR-VP16$^{+/+}$) have been particularly informative in defining the roles of murine and human PXR in the liver (12, 69). The results indicate that PXR has a key role in protecting mice from xenobiotic toxicants (69) or from hepatotoxic bile acids (12, 13). Presumably, PXR regulates the expression of hepatic genes that are involved in clearance of toxic compounds, such as LCA, from the blood and their subsequent metabolism and excretion into the bile (Fig. 2). Recently, we identified one gene (MRP2; ABCC2) involved in this excretion process that, surprisingly, is activated by PXR, FXR, and CAR (constitutive androstane receptor) (22a). This latter result emphasizes the overlapping roles of multiple nuclear receptors.

3. Macrophages

Macrophages express LXRα and LXRβ. In contrast, neither FXR nor PXR appear to be expressed in this cell type. Incubation of human or murine macrophages with either oxysterols or synthetic LXR agonists results in increased expression of ABCA1 (70–72), ABCG1 (73), apolipoprotein E (22), and LXRα (74, 75). Functional LXREs have been identified in the promoters or enhancers of each of these genes (Fig. 1). Interestingly, LXR induces the LXRα gene itself in human (but not murine) macrophages by a process that is dependent on an LXRE in a distal enhancer (74, 75) (Figs. 2, 3). In addition, the LXRα gene is induced by PPARγ and PPARα (76–78) that may in turn be activated by the newly synthesized unsaturated fatty acids.

The LXR-dependent increased expression of ABCA1 has been shown to function in the increased efflux of phospholipid and cholesterol from the cells to exogenous lipid-poor proteins (Fig. 3) (79, 80). Such acceptors include apolipoprotein A1 and apolipoprotein E (81, 82). Since apoE expression is increased in macrophages following activation of LXR, the secreted apoE protein may function as an acceptor for effluxing phospholipids and cholesterol (83). Recent studies indicate that ABCA1 may mediate the efflux of cellular phospholipids to extracellular protein acceptors as the primary event (84, 85), and that cellular cholesterol, possibly derived from late endosomes/lysosomes, subsequently effluxes to the preformed extracellular phospholipid/protein complex (86, 87).

Our current ideas about the importance of ABCA1 come from the observations that HDL is virtually absent from the blood of patients or mice containing mutations in both ABCA1 alleles. The demonstration that transgenic mice, expressing the human ABCA1 gene from a BAC clone, have elevated HDL levels (88) provides additional evidence that the ABCA1 protein has a critical role in controlling HDL levels.

In contrast to ABCA1, there is much less information on the function of ABCG1. The results of studies with cultured cells treated with antisense oligonucleotides to ABCG1 suggest that this protein may be involved in controlling the efflux of cellular cholesterol to HDL and/or the secretion of apoE (89, 90). ABCG1 has been reported to reside in the endoplasmic reticulum and Golgi membranes (89). However, recent studies have reported that multiple ABCG1 transcripts are produced as a result of the use of alternative promoters and alternative RNA splicing; translation of these transcripts produces multiple ABCG1 proteins with significantly different amino termini (Fig. 3) (91, 92). It is possible that these alternative forms of the ABCG1 protein combine to form different heterodimers with related transport functions. Consequently, additional studies will be required to determine both the tissue and cellular location and function of each of these ABCG1 isoforms.

4. The role of LXR and FXR in lipoprotein metabolism

A. Role of LXR in lipoprotein metabolism. As discussed above, plasma lipid levels are also modulated by LXR agonists, presumably in part as a result of the activation of SREBP-1c and subsequent increases in the rate of synthesis of fatty acids, triglycerides and phospholipids (Fig. 2). In addition, increased expression of ABCA1 and ABCG5/ABCG8 presumably alters sterol absorption and the efflux of cellular cholesterol and phospholipid from liver and macrophages into the plasma or bile. (Figs. 2, 3). In addition, CETP and lipoprotein lipase (LPL) genes are also directly activated by oxysterols and LXR (93, 94). Since CETP functions to facilitate the transfer of cholesteryl esters between plasma lipoproteins, and while LPL catalyses the hydrolysis of lipoprotein triglycerides, it is clear that LXR modulates lipoprotein metabolism (Figs. 2, 3). Interestingly, the expression of apolipoprotein C-II (apoC-II), the obligate cofactor for LPL, is induced by FXR (Figs. 1–3) (95). We would predict that additional LXR target genes will be identified that directly control different aspects of lipoprotein metabolism.

B. Role of FXR in lipoprotein metabolism. Based on studies with FXR null mice and the identification of a limited number of FXR target genes, it has become evident that this nuclear receptor controls specific aspects of lipoprotein metabolism. The idea that FXR might have such a role came when the second FXR target gene was identified and shown to encode PLTP(phospholipid transfer protein)

(22, 96). PLTP is a secreted protein that facilitates the transfer of phospholipids and cholesterol from triglyceride-rich lipoproteins to HDL and consequently modulates the concentration of plasma HDL (Figs. 1–3) (97). More recent studies that utilized FXR-overexpressing cells and suppression subtraction hybridization identified apoC-II as an additional FXR target gene (Figs. 1–3) (95). Induction of hepatic apoC-II by activated FXR was dependent on the presence of IR-1 elements (FXRE/BARE) (95) contained within two distal enhancers that have been termed Hepatic Control Regions (HCR.1 and HCR.2) (98). Interestingly, these 319 bp HCRs are located within 11 kb and 22 kb 5′ of the apoC-II gene transcriptional start site, and are critical for the liver specific expression of a number of genes including apoC-II (99). Preliminary studies also show that hepatic apoE mRNA levels also increase following the activation of FXR by bile acids (Kast et al., unpublished observations). Since apoE is located in a gene cluster that includes apoC-I, apoC-II, and apoC-IV (99), we hypothesize that the FXREs/BAREs in HCR.1 and HCR.2 may be involved in the regulated transcription of multiple apolipoproteins. As PLTP, apolipoprotein C-II and apolipoprotein E are all secreted into the blood and are known to be involved in the metabolism of plasma lipoproteins, these data suggest that bile acid-activated FXR has a central role in regulating plasma lipid levels (Figs. 2, 3). Consistent with this proposal, the administration of either a synthetic FXR ligand (GW4064) (100) or a natural FXR ligand (cholic acid) (95) to rodents results in a significant decline in plasma triglyceride levels. The finding that administration of a cholesterol-rich diet to FXR null mice results in elevated levels of proathergenic lipoproteins (61) provides additional support for the idea that FXR has an important role in controlling normal plasma lipoprotein levels.

SUMMARY

It has been less than four years since natural ligands for LXR, FXR, and PXR were identified. The rapid increase in our understanding of these three receptors in this short time is a direct result of finding the "holy grail" (i.e., the natural ligands) and generating mice in which the genes encoding LXR, FXR, or PXR have been deleted. The data from these studies are consistent with the proposal that, under a variety of dietary conditions, the control of lipid homeostasis is dependent upon cross talk between these three nuclear receptors. For example, excess uptake of cholesterol from the diet results in the hepatic production of oxysterols, activation of LXR, increased expression of CYP7A1, and enhanced production of primary bile acids. In turn, bile acid-activated FXR enhances the expression of genes involved in bile acid excretion (BSEP) and reabsorption (I-BABP), while inhibiting the expression of additional CYP7A1 (via SHP). At the same time, hepatic PXR, activated by LCA generated in the intestine, represses CYP7A1 and activates genes involved in bile acid metabolism (CYP3A).

In addition, the studies cited in this review demonstrate that the metabolism of plasma lipoproteins is also significantly affected by these nuclear receptors as a result of the increased expression of apoC-II, apoE and PLTP (by FXR), and CETP and LPL (by LXR). Despite these new insights into the roles of LXR, FXR, and PXR, and despite the spectacular scientific inroads that have recently been made, it is quite clear that our knowledge is far from complete. The future is likely to be no less exciting and stimulating as investigators attempt to unravel the roles of LXR, FXR, and PXR as regulators of lipid metabolism.

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Characterization of the cocaine- and amphetamine-regulated transcript (CART) peptide gene promoter and its activation by a cyclic AMP-dependent signaling pathway in GH3 cells

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Abstract

Cocaine- and amphetamine-regulated transcript (CART) peptides are regulated neuropeptides that play a role in a variety of physiological processes. CART mRNA is also highly regulated as its levels change in response to psychostimulant drugs and leptin. To understand the mechanisms involved in regulating CART mRNA levels, the mouse CART 5′-flanking regulatory region was studied. The sequence of 3.4 kb of the mouse CART 5′-flanking region revealed a proximal promoter that contains a cluster of transcription factor binding sites, including an overlapping STAT/CRE/AP1 site. In addition, the 5′-most 320 bp of the CART promoter shares 83% nucleotide identity between mouse and human. Three luciferase expressing constructs containing varying amounts of CART 5′ upstream sequence were generated and tested for promoter activity. Transient transfection of GH3 cells with constructs containing 641 and 3451 bp of upstream sequence displayed strong promoter activity, producing 29-fold and 51-fold stimulation, respectively, while, a construct containing 102 bp of upstream sequence displayed a 5.4-fold increase in activity. A construct containing the composite STAT/CRE/AP1 site was responsive to cyclic AMP induction by forskolin in GH3 cells. Forskolin treatment also resulted in a 4.5-fold increase in CART mRNA levels after 6 h and the addition of H89, an inhibitor of protein kinase A, reduced the levels by 50%. These studies indicate that the CART proximal promoter lies within the 5′-most 641 bp and that in GH3 cells the CART gene is regulated via a cyclic AMP-dependent pathway.

Keywords: AP1, CART, CRE, cyclic AMP, PKA, promoter.

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CART (cocaine- and amphetamine-regulated transcript) is a highly regulated mRNA, and CART peptides are regulatory peptides that are expressed only in neurons and not other cells in the nervous system. CART mRNA expression is regulated by psychostimulant drugs (Douglass *et al.* 1995; Fagergren and Hurd 1999; Brenz Verca *et al.* 2001) as well as by leptin (Kristensen *et al.* 1998; Wang *et al.* 1999; Ahima and Hileman 2000; Dhillon *et al.* 2000) and presumably by other stimuli. CART mRNA and its cleaved peptides are widely distributed in the brain and other endocrine tissues, including the pituitary and adrenals (Douglass *et al.* 1995; Koylu *et al.* 1997). CART peptides are thought to have a role in multiple physiological functions, including reward and reinforcement, feeding, stress, autonomic and endocrine control, and sensory processing (Kuhar and Dall Vechia 1999; Thim *et al.* 1999; Kuhar *et al.* 2000). Characterization of the mechanisms involved in CART gene regulation will aid in understanding CART peptides' homeostasis.

The rat, human, and mouse CART genes have been identified and sequenced (Douglass and Daoud 1996; Douglass *et al.* 1995; Adams *et al.* 1999). The CART gene is composed of three exons and two introns, with rat and mouse having alternatively spliced variants (Douglass *et al.* 1995; Adams *et al.* 1999). Splicing occurs within exon 2 of the long form, which results in a transcript that is missing 39 nucleotides (termed the short form). Also, in rats, two major CART mRNA species have been described which are the results of differential usage of polyadenylation sites.

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Abbreviations used: CART, cocaine- and amphetamine-regulated transcript; MCH, melanin-concentrating hormone; PKA, protein kinase A; POMC, proopiomelanocortin; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate buffer; TRH, thyrotropin-releasing hormone.

Our laboratory has started to address the molecular mechanisms involved in the regulation of CART mRNA expression. The data presented identifies putative *cis*-regulatory elements, and functionally tests various segments containing these *cis*-regulatory elements for promoter activity. The role of the cyclic AMP signal transduction pathway was also examined.

Experimental procedures

Tissue culture and stimulation

Rat pituitary GH3 and rat pheochromocytoma PC12 cells were maintained in Ham's F-12 media supplemented with 15% horse serum and 5% fetal bovine serum (Life Technologies, Rockville, MD, USA). Mouse pituitary AtT20 cells were maintained in Dulbecco's minimal essential media supplemented with 10% horse serum (Life Technologies). All tissue cultures were maintained in a humidified incubator at 37°C under 5% CO_2.

GH3 cells were grown in poly-L-lysine-coated 10 cm cell culture plates to 70–80% confluency. Media was then changed to Ham's F-12 supplemented with 0.5% horse serum for 16–24 h. In the some experiments, following serum deprivation, new 0.5% horse serum supplemented media was added containing 30 μM *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) (Sigma, St Louis, MO, USA) for 2 h followed by addition of 20 μM forskolin (Sigma) to the media for 1, 3, 6, 12, and 24 h. Similarly, samples were treated with forskolin in the absence of H89.

RNA isolation and northern blot

Total RNA was isolated from frozen rat cerebellum and hypothalamus or GH3 cells. Tissues (50–100 mg) were homogenized in 1 mL of Trizol Reagent (Life Technologies, Grand Island, NY, USA) using a power homogenizer. GH3 cells were lysed in 1 mL of Trizol Reagent per 1 cm^2 of culture dish surface area. Samples were incubated in Trizol Reagent and RNA isolated according to the manufacturer's recommendation.

Total RNA was run on a 1.2% 3-[*N*-morpholino]propanesulfonic acid (MOPS, Sigma)/6% formaldehyde agarose gel. RNA was transferred by capillary action in the presence of 10 × saline sodium citrate buffer (SSC; 1.5 M NaCl 0.15 M sodium citrate, pH 7.0) and fixed by UV cross-linking. Prehybridization was carried out at 50°C in 6 SSC, 5 × Denhardt's solution (Sigma), 1% sodium dodecyl sulfate (SDS), 20% formamide, 50 μg/mL sonicated salmon sperm DNA (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) for 6 h. Hybridization buffer was changed and 2×10^6 cpm of a ^{32}P-labeled cocktail consisting of two or three oligonucleotides was added and incubated at 50°C for 24 h. Oligonucleotides were 5′ end labeled using γ-^{32}P (6000 Ci/mmol) and polynucleotide kinase (Stratagene, La Jolla, CA, USA). The CART cocktail consisted of three oligos with the following sequences: 5′-TGAAAACAAG-CACTTCAAGAGGAAAG-3′, 5′-TGCAACGCTTCGATCTGCA-ACATAG-3′, and 5′-CTCATGCGCACTCTCTCCAGCG-3′. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was made up of two oligos having the following sequences: 5′-AGT-TGTCATTGAGAGCAATGCCAGC-3′ and 5′-AGTAGACTCAC-GACATACTCAGCA-3′.

Nonspecific hybridization was removed by washing the blots with increasing stringency washes (5 × SSC, 1% SDS up to 2 × SSC, 0.1% SDS) at room temperature. Blots were analyzed by autoradiography or using a PhosphoImager (Storm, Molecular Dynamics, Sunnyvale, CA, USA). Blots were stripped and re-probed with the GAPDH probe. CART mRNA level was normalized to GAPDH RNA.

Sequencing and analysis of the mouse CART gene promoter region

A previously described mouse BAC genomic library was screened using a rat CART cDNA probe. One hybridization positive clone (approximately 125 kb in length) was identified (Adams *et al.* 1999). A *Bam*HI subclone, Bam5′C (approximately 9.5 kb in length), containing the entire CART gene was generated (Fig. 1). The CART 5′-flanking region in Bam5′C (*Bgl*II/*Sac*I segment) was sequenced. All sequences were determined by cycle-sequencing reactions using a dye-labeled chain terminator sequencing kit. Cloned fragment termini were sequenced using vector-derived primers; custom designed primers were used to walk across the region until double-stranded coverage across the region was obtained with a four-fold redundancy or greater. The sequence has been deposited with GenBank under accession number AF148071.

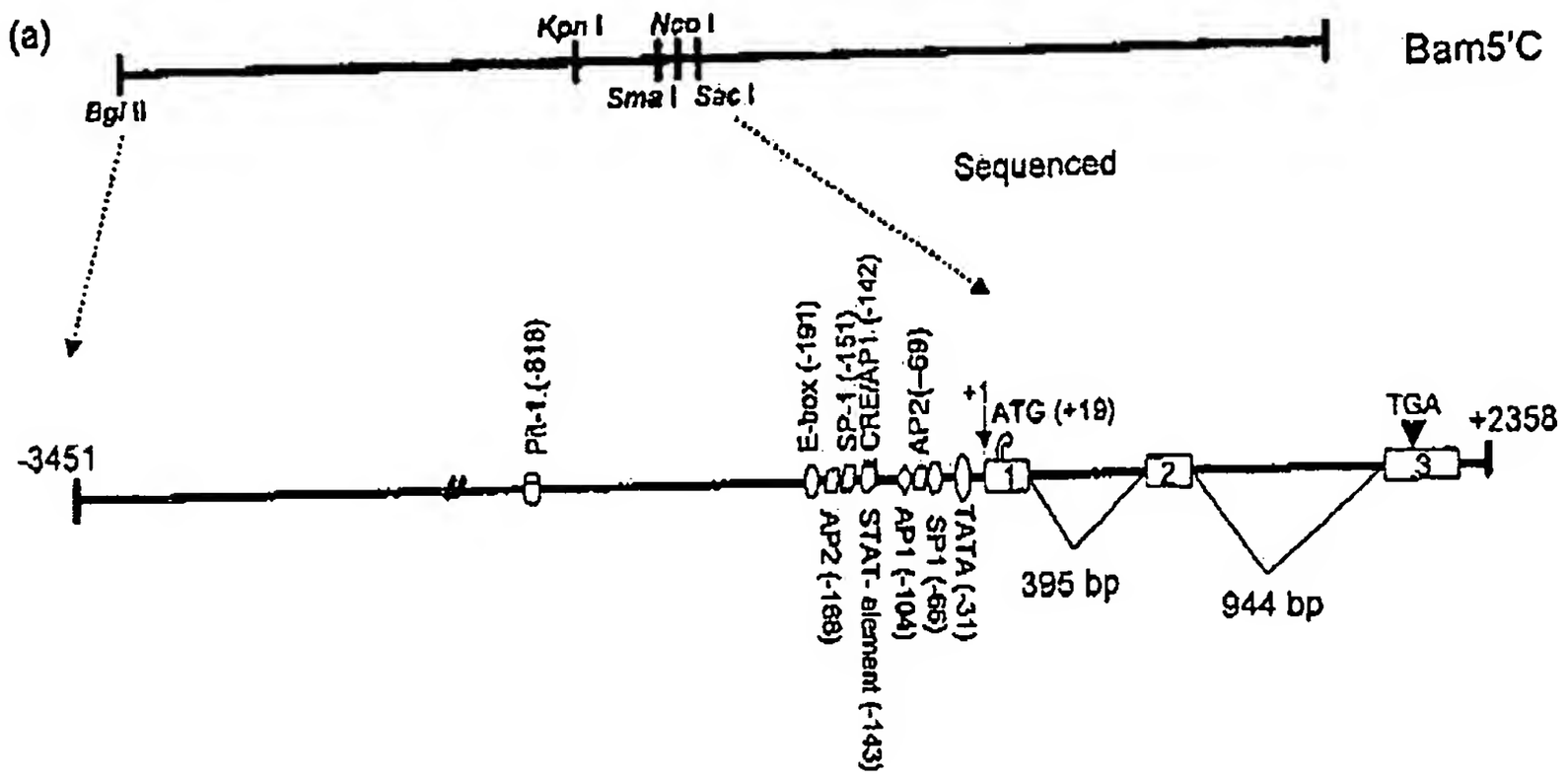
The sequenced region was analyzed for putative transcription factor binding sites using the Transcription Factor Database (TRANSFAC) and MatInspector V2.2 (Wingender *et al.* 2000). The sequence was also analyzed for specific patterns previously shown to be involved in restricting gene expression to a particular cell type using FindPatterns in the Wisconsin Package (Genetics Computer Group, Madison, WI, USA).

Cloning

Various lengths of 5′ upstream sequences were cloned into the promoter-less vector, pGL3-BASIC (Promega, Madison, WI, USA), and these clones were tested for promoter activity. Constructs were generated by digesting the genomic clone, Bam5′C, with *Bgl*II/*Nco*I, or *Kpn*I/*Sac*I, or *Sma*I/*Nco*I. pGL3-BASIC vector was digested and dephosphorylated followed by ligation to the appropriately digested genomic fragment using T4 DNA Ligase (Promega). Reactions were incubated overnight at 4°C and used to transform competent *Escherichia coli* TOP10 cells (Invitrogen, Carlsbad, CA, USA). Representative colonies were picked and plasmid DNA isolated. Constructs were confirmed by dideoxy nucleotide sequencing (Emory DNA Sequencing Core Facility). The three pGL3-luciferase expressing constructs made were: −3451CART-LUC, spanning −3451 to + 23; −641CART-LUC, spanning −641 to + 30; and −102CART-LUC, spanning −102 to + 23 where + 1 is the predicted site of transcription initiation.

Transfection and luciferase assay

GH3, AtT20, and PC12 cells were plated on 35-mm poly L-lysine-coated six-well plates at a density of 1×10^6 cells/well in 2 mL of fully supplemented media as described. For each transfection, 2 μg of pSV-β-Galactosidase Vector (Promega), 2 μg of one of the pGL3-luciferase expressing constructs, and 6 μL of FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA) were mixed in the appropriate serum free media and



(b)

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          -320
Human     GAAGGCATTT TCCATTtcct aggcccctcc cgcttcccgT CCCTCACCTc TaGCCCCTCg
Mouse     GAAGGCATTT TCCATT.... .......... ..........T CCCTCACCTt TtGCCCCTCa

          -260
Human     GcTTCAGCTC aGGCCCCTcG GGgAGCaTCC CTtGCcGtGa GACtGACAGC cTTTgGGGGC
Mouse     GtTTCAGCTC tGGCCCCTaG GGaAGCtTCC CTgGCtGcGg GACgGACAGC gTTTcGGGGC

          -200                                E-box                    AP2
Human     gCaGGGTCcT GTTcTCTGCG CTCTAGCCCA TCTGTGCGCA GAGCCTCGTT CCCaGGCgCC
Mouse     aCgGGGTCtT GTTtTCTGCG CTCTAGCCCA TCTGTGCGCA GAGCCTCGTT CCCgGGCtCC

          -160     SP1     STAT/CRE/AP1                                AP1
Human     tGGAaCCCGG CGGGCATTGA CGTCAAgCGc CgGCGGAGCG CTGCCTACAG ACGGtTGACC
Mouse     cGGAgCCCGG CGGGCATTGA CGTCAAaCGg CaGCGGAGCG CTGCCTACAG ACGGcTGACC

          -100                                      SP1
Human     CGGGCcCTCC TCCACACCCC CtTCCTTCTT CgCCtCCTCC CTCTTtCCtG CACggGggcT
Mouse     CGGGCtCTCC TCCACACCCC CgTCCTTCTT CtCCcCCTCC CTCTTcCCgG CACccGattT

          -40      TATA-box          +1                    22
Human     CgggCtcaCT ATAAaAgGtg GGAGcGCGtg GTGCCCcAGC AaCGAcGAGt TtCAGAACgATG
Mouse     C.aaCcggCT ATAAgAaGcc GGAGaGCGca GTGCCCgAGC AgCGAgGAGg TcCAGAACcATG
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Fig. 1 Structure of the mouse CART gene and sequence of the proximal promoter region. (a) A diagram of the region in Bam 5'C (see Experimental procedures) that was sequenced. The genomic structure of the CART gene as found in the clone is shown. The three exons and two introns of the CART gene are indicated. Exons are drawn as clear numbered boxes and the length of the introns is given. The cluster of transcription factor binding sites is shown and each is represented by a unique symbol. The diagram is not drawn to scale. The location of each transcription binding site relative to the site of transcription initiation (+ 1) is given in parenthesis. (b) Sequence alignment between the human and mouse proximal promoter region. Differences between human and mouse are in lower case. The transcription binding sites are underlined. The predicted site of transcription initiation is indicated as + 1 and the initiating ATG is given. See text for additional details.

incubated at 25°C for 45 min as described by the manufacturer. One hundred microliters of the complexed DNA/FuGENE 6 mixture was added to each well and incubated for 18 h at 37°C under 5% CO_2. In some cases, media was replaced with 2 mL of supplemented media containing 25 μM forskolin and incubated for additional amounts of time. Cells were lysed and luciferase expression measured using the Luciferase Assay System as recommended by the vendor (Promega).

Luciferase activity quantified using a luminometer (Turner Designs Model TD-20/20 luminometer, Sunnyvale, CA). β-Galactosidase

activity was measured using the β-Galactosidase Enzyme Assay System (Promega) and the absorbance read at 420 nm. Protein concentration was determined using the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA). In all experiments, luciferase expression was normalized to β-galactosidase activity using equal protein concentration.

Measurements were carried out on three or more independent transfected cultures that were done in duplicate. All results were expressed as mean ± SEM. Statistical significance was determined using one-way ANOVA followed by a Tukey test or a Student's *t*-test (SigmaStat 2.0, Jandel Corporation).

Results

CART's proximal promoter region contains transcription factor binding sites that are conserved between mice and humans. Analysis of the mouse CART gene 5′ upstream sequence identified a region containing a cluster of transcription factor binding sites, including a canonical cyclic AMP response element (CRE), two AP1 and SP1sites, and several AP2 sites, along with a TATA-like sequence and an E-box (Fig. 1). There is also a STAT-response element, TT $(N)_6$AA, in an overlapping STAT/CRE/AP1 site. Further upstream there is a putative binding site for the pituitary-specific transcription factor Pit-1, AWWTATNCAT, where W is either an A or a T (Fig. 1a).

The CART gene upstream sequence was further analyzed using a neural network promoter-predicting algorithm (Ohler *et al.* 1999) in order to predict transcription initiation sites for the mouse CART gene. Transcription initiation for the mouse CART gene was predicted at the A located 19 nucleotides upstream of the initiating AUG codon (Fig. 1b). This is in agreement with the previously published rat and human 5′ ends (Douglass *et al.* 1995; Douglass and Daoud 1996).

The CART peptide coding sequence is highly conserved between rodents and man, with greater than 90% nucleotide identity (Douglass and Daoud 1996). To determine if the 5′-flanking region of the mouse CART gene was similarly conserved across species, the human CART 5′-flanking sequence was identified in the databases. A contig (Accession Number NT019389) located on human chromosome 5 and spanning 877,249 bp was found. It contained the CART gene plus 5′ upstream sequence. A pairwise comparison between the mouse and human sequences was performed using GAP (GCG Wisconsin Package; Accetys Inc., San Diego, CA, USA) with a gap weight of 50 and length weight of 3. Figure 1(b) is an alignment of the mouse proximal promoter region with the corresponding human region. The comparison identified a 320-bp region immediately upstream from the CART coding sequence that shares 83.4% nucleotide identity. The region contains the binding sites for the clustered set of transcription factors, including the overlapping CRE/AP1/STAT site.

Functional studies

To assess the importance of the regulatory elements within the CART upstream sequence, the activity of various 5′-proximal regions of the CART gene were tested for their ability to drive gene expression when cloned upstream of the luciferase gene. Three luciferase expressing constructs were made and tested; −3451CART-LUC, −641CART-LUC, and −102CART-LUC that contain 3451, 641, and 102 bp of mouse CART 5′ upstream sequence (+1 is the predicted site of transcription initiation), respectively (Fig. 2). We chose GH3 cells as our *in vitro* system because CART mRNA is expressed in the pituitary (Couceyro *et al.* 1997) and these cells had previously been shown to express high levels of CART mRNA (Barrett *et al.* 2001). GH3 cells were cotransfected with pSV-β-galactosidase (which serves as an internal control for normalization of transfection efficiency)

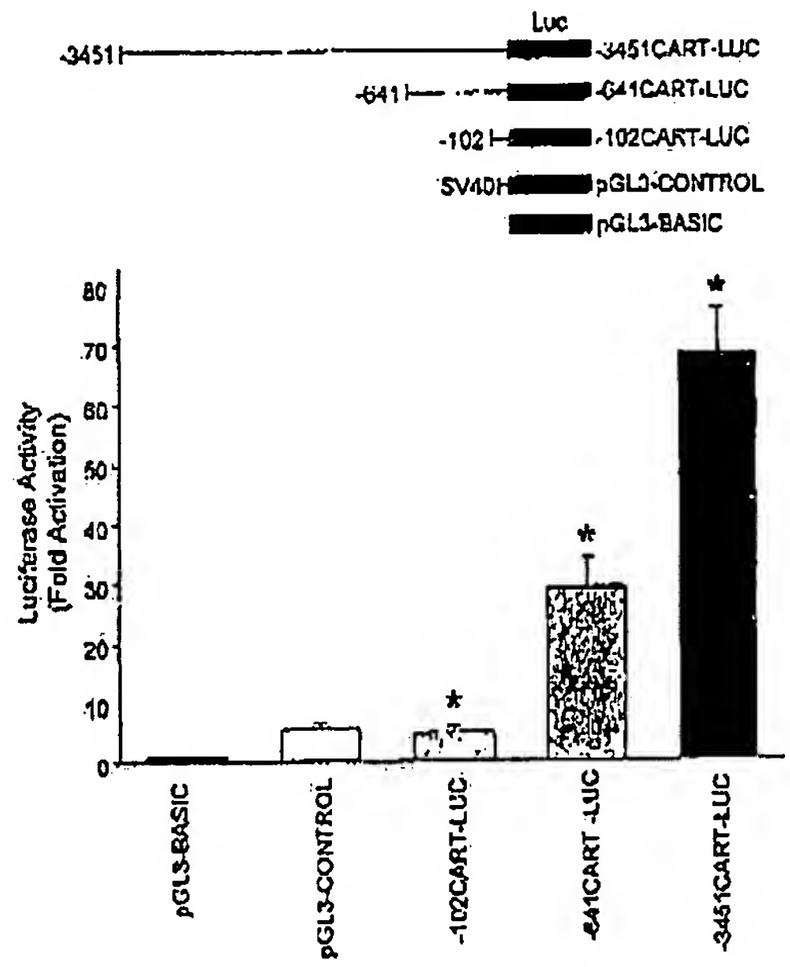

Fig. 2 Luciferase (LUC) expression of constructs carrying varying lengths of the CART promoter. The constructs were cloned into pGL3-BASIC vector which lacks a promoter, and the luciferase-expressing clones were transiently cotransfected with β-galactosidase vector, which serves as an internal control for normalization of transfection efficiency, into GH3 cells. Boundaries for the CART-LUC constructs are: −3451CART-LUC, −3451 to +23; −641CART-LUC, −641 to +30; −102CART-LUC, −102 to +23 where +1 is the site of transcription initiation (see Fig. 1b). pGL3-CONTROL contains the SV40 promoter to drive LUC expression and serves as a positive control. Each value is expressed as the folds increase relative to that found with pGL3-BASIC. Values are mean ± SEM from at least three independent experiments each done in duplicate. Statistical significance (*) was assessed using one-way ANOVA followed by a Tukey test. The differences in the values among the different constructs are greater than would be expected by chance ($p = < 0.001$).

and one of the CART containing constructs, −3451CART-LUC, −641CART-LUC, and −102CART-LUC. Luciferase activity of each construct was compared to that produced by pGL3-BASIC, the parent vector that lacks a promoter (background activity).

pGL3-CONTROL, which has a SV40 promoter to drive luciferase expression, was used as a positive control since it exhibits strong promoter activity in a variety of mammalian cells. In GH3 cells, pGL3-CONTROL had a six-fold increase in luciferase activity above pGL3-BASIC. This is similar to the 5.4-fold activation by −102CART-LUC, indicating that this segment of upstream sequence can function as a promoter and drive luciferase expression. However, constructs containing larger upstream segments produced much greater activity. −641CART-LUC produced a 29-fold activation and −3451CART-LUC had a 51-fold activation. The −102CART-LUC construct does not contain the E-box, AP2, SP1, or STAT/CRE/AP1 sites highlighted in Fig. 1(b) but it does contain the TATA-like box and an SP1 site. The −641CART-LUC construct contains the STAT/CRE/AP1 composite site, and −3451CART-LUC also contains a putative Pit-1 binding site. Pit-1 is a transcription factor belonging to the POU domain proteins that is expressed exclusively in the central nervous system and in the pituitary (reviewed by McEvilly and Rosenfeld 1999).

Cyclic AMP-dependent activation of the CART gene in GH3 cells

Because these promoter studies were carried out in GH3 cells, it is important to show that these cells normally express CART mRNA. Barrett *et al.* (2001) have shown that CART mRNA levels were up-regulated by activators of cyclic AMP, including forskolin, dibutryl-cyclic AMP, and PACAP-38 in these cells. To confirm and extend their results, a time course of forskolin induced activation of CART mRNA was performed. Forskolin is an activator of adenylate cyclase, which is the enzyme that converts adenosine triphosphate to cyclic adenosine monophosphate. GH3 cells were treated with 20 μM forskolin for 0, 1, 3, 6, 12, and 24 h as described in Experimental procedures and 40 μg of total RNA was analyzed by northern blot analysis (Fig. 3). Continuous exposure to forskolin increased CART mRNA levels compared to 0 h of exposure. The involvement of protein kinase A (PKA) was also tested (Fig. 3). In the same experiment, some GH3 cells were treated with 30 μM H89, an inhibitor of PKA, for 2 h prior to and during forskolin treatment. Pretreatment of PC12 cells with the same concentration of H89 results in a significant reduction of forskolin-induced protein phosphorylation (Chijiwa *et al.* 1990). The time course showed that the maximal increase in CART mRNA levels occurred after 6 h of forskolin treatment. Because it is well established that forskolin increases cFos mRNA (reviewed by Herdegen and Leah 1998), the effect of forskolin on cFos expression in GH3 cells under the same experimental conditions was tested; maximal expression was seen after 30 min, after which time levels decreased (data not shown). Treatment with H89 prior to and during forskolin treatment reduced the CART mRNA levels by approximately two-fold.

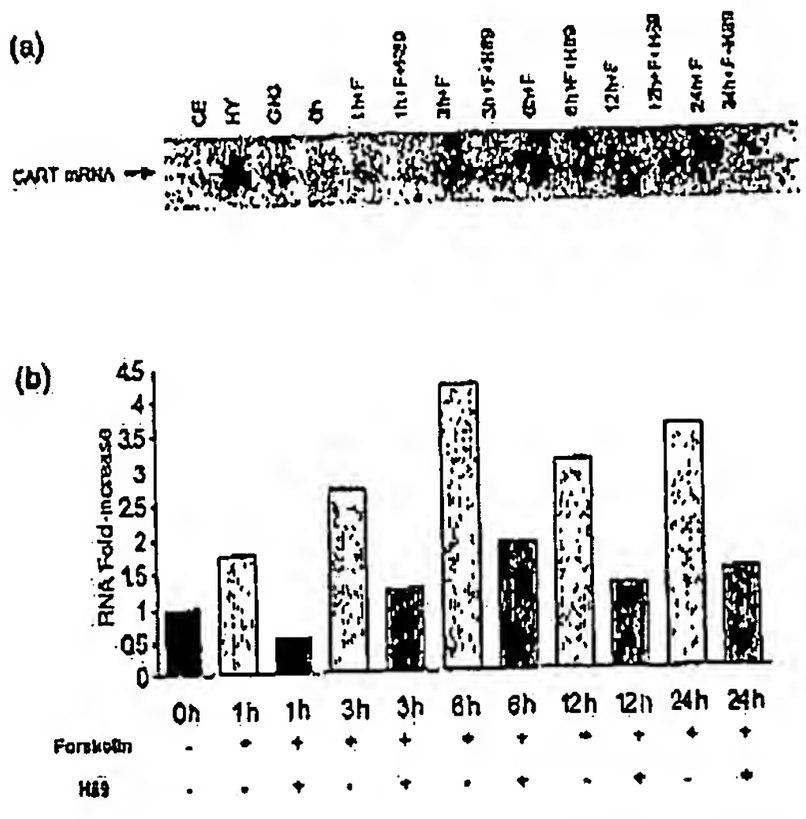

Fig. 3 Changes in CART mRNA levels in response to forskolin (F) and H89 treatments. (a) Northern blot analysis of CART mRNA levels in brain regions and GH3 cells treated with forskolin (F) alone or F plus H89 as described in Experimental procedures. The 0 h time point shows CART mRNA in the absence of any treatment. Total RNA (40 μg) was transferred by capillary action and probed with a CART oligonucleotide cocktail. Brain regions analyzed: CE, cerebellum as a negative control (20 μg); and HY, hypothalamus as a positive control (20 μg). (b) CART mRNA levels were quantified and normalized to GAPDH levels. RNA fold increase was determined by setting the 0 h time point to 1 and comparing the other time points to the 0 h time point. Data is from a single experiment but is representative of data from three independent experiments.

To determine which *cis*-elements present in the CART promoter were responsible for the responsiveness to cyclic AMP induction by forskolin, two luciferase expressing constructs, −641CART-LUC which contains the clustered transcription factor binding sites including the overlapping STAT/CRE/AP1 site, and −102CART-LUC which lacks the STAT/CRE/AP1 site were tested. The −641CART-LUC and −102CART-LUC constructs were transfected into GH3 cells and 18 h after transfection, media was changed and cells were treated with 25 μM forskolin for 7 h. As illustrated in Fig. 4, a two-fold increase in luciferase activity after forskolin treatment was produced with −641CART-LUC, while no statistically significant increase in luciferase activity was observed with −102CART-LUC and pGL3-CONTROL. This suggests that the region between −102 and −641 contains *cis*-elements that are responsive to changes in cyclic AMP levels.

A comparison of luciferase activity between −641CART-LUC and pGL3-CONTROL after forskolin treatment showed

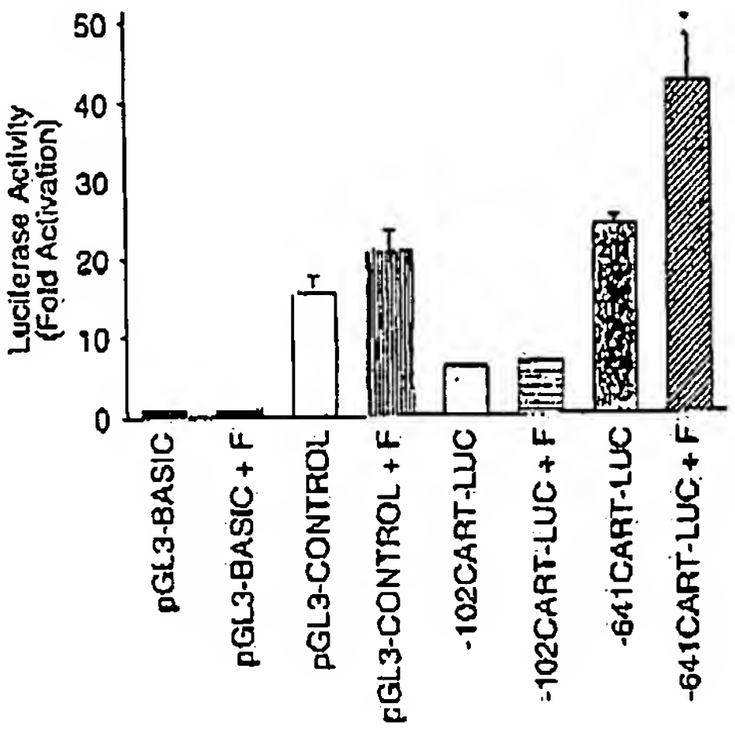

Fig. 4 Cyclic AMP responsiveness of −102CART-LUC and −641 CART-LUC in GH3 cells. GH3 cells were transiently transfected with each construct as described in Experimental procedures and luciferase activity assayed 7 h after forskolin (F) treatment. Samples are compared to the corresponding nontreated control. Each value is expressed as the folds increase relative to pGL3-BASIC. Values are mean ± SEM from at least three independent experiments each done in duplicate. Statistical significance (*) was assessed using a student t-test. The difference between the values of −641CART-LUC and −641CART-LUC + F is significant ($p = 0.037$).

that −641CART-LUC exhibits a continuous increase in expression beginning at 1 h and continuing through 48 h (Fig. 5). In comparison, pGL3-CONTROL exhibited greatest luciferase activity after 7 h of forskolin treatment, with a three-fold decrease after 24 h.

Cyclic AMP responsiveness of −641CART-LUC in cells of different origins. Promoter activity of −641CART-LUC in AtT20 and PC12 cells was examined. AtT20 and PC12 cells were transfected as described in Experimental procedures.

−641CART-LUC was more active in PC12 cells than in AtT20 cells (Fig. 6) however, promoter activity was highest in GH3 cells (29-fold above background, Fig. 2). Forskolin treatment of AtT20 and PC12 cells did not have a significant effect on luciferase expression. In addition, in AtT20 cells, increasing the amount of 5′ upstream sequence by using −3451CART-LUC did not significantly affect the luciferase expression level in the presence or absence of forskolin treatment, which is contrary to what we observed in GH3 cells (data not shown). CART mRNA is expressed at much lower levels in AtT20 cells than in GH3 cells (Dominguez G., unpublished result and Barrett *et al.* 2001).

Discussion

CART mRNA levels change in response to various stimuli including fasting, leptin, and psychostimulant drugs (Douglass *et al.* 1995; Kristensen *et al.* 1998; Fagergren and Hurd 1999; Hurd *et al.* 1999; Wang *et al.* 1999; Abima *et al.* 2000; Dhillon *et al.* 2000), but the transcription factors involved in CART mRNA regulation have not been studied. The aim of this work was to characterize the CART promoter by identifying putative transcription factor binding sites and to test segments containing these elements for functional promoter activity.

Sequence analysis of a 3.4-kb mouse genomic DNA identified a 320-bp region, located immediately upstream from the transcriptional start site, that is highly conserved

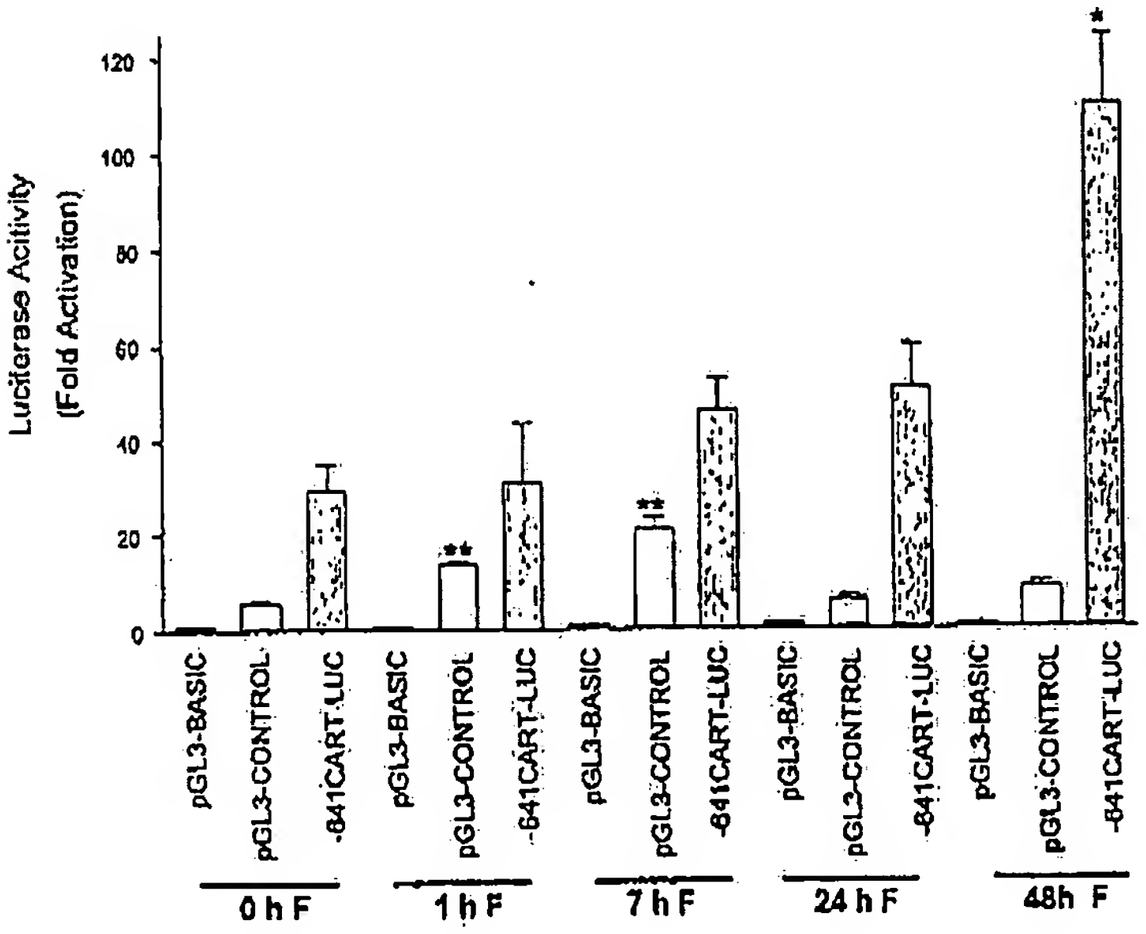

Fig. 5 Time course of forskolin (F) effect on −641CART-LUC in GH3 cells. Cells were treated with forskolin (25 μM) for 1, 7, 24, and 48 h. Each value is expressed as the folds increase relative to pGL3-BASIC. Values for pGL3-CONTROL are included for comparison. Values are mean ± SEM from at least three independent experiments each done in duplicate. Statistical significance (*) was assessed using one-way ANOVA followed by a Tukey test. The differences between the values of −641CART-LUC at 48 h F and the other time points are greater than would be expected by chance ($p = < 0.001$). **Indicates that the increase in the values of pGL3-CONTROL at 1 h and 7 h are significantly different than the 0 h time point ($p = < 0.05$).

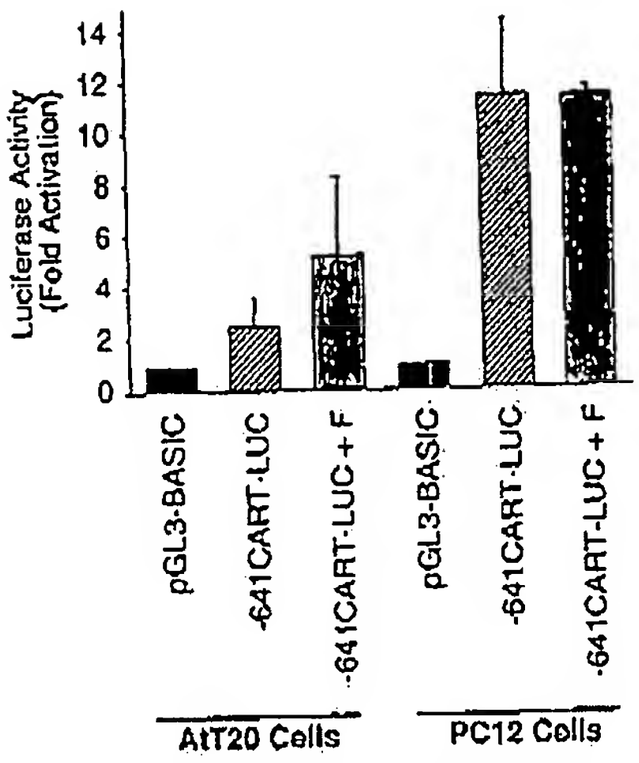

Fig. 6 Comparison of the activity of −641CART-LUC in AtT20 and PC12 cells in the presence and absence of forskolin (F). AtT20 and PC12 cells were transiently transfected with −641CART-LUC as previously described. Luciferase activity is expressed as the folds increase relative to pGL3-BASIC.

between the mouse and human CART gene (Fig. 1b). The region contains several transcription factor binding sites and thus suggests that the mouse and human CART gene could be transcriptionally regulated by the same transcription factors. For example, the overlapping STAT/CRE/AP1 site located 148 nucleotide upstream from the predicted site of transcription initiation is completely conserved between mouse and human. Additionally, the conservation of these binding sites suggests that these transcription factors have an important role in the regulation of the CART gene.

Transcriptional activation of cellular genes by a cyclic AMP-responsive element (CRE)-binding protein (CREB)-mediated response usually peaks after 30 min of stimulation and decreases after 2–4 h (reviewed by Mayr and Montminy 2001), the continuous increase that we observed in GH3 cells (Fig. 5) suggests that the cyclic AMP responsiveness of the CART promoter may not be due only to CREB-mediated activation but to activation by other transcription factors. Theoretically, the STAT/CRE/AP1 element could allow complex transcriptional regulation via CREB and cJun proteins as well as by signal transducer and activator of transcription (STAT) proteins (Shaywitz and Greenberg 1999). Composite CRE/AP1 sites have been found in a number of genes involved in neurotransmitter synthesis, including dopamine β-hydroxylase (Shaskus *et al.* 1992), prodynorphin (Messersmith *et al.* 1996), proenkephalin (Comb *et al.* 1986), and cholecystokinin (Hansen *et al.* 1999). For example, the inflammation-induced model proposed by Messersmith *et al.* (1998) for prodynorphin gene transcription describes the interaction between Fos/Fra, phosphorylated-CREB, and phosphorylated-cJun at the composite DYNCRE3 site. Mutational analysis of the STAT/CRE/AP1 composite site will be very important in identifying which transcription factors are involved in the cyclic AMP responsiveness of the CART promoter.

The presence of a STAT-binding motif suggests that the CART gene could be regulated directly via cytokine signaling. This could be a mechanism by which leptin stimulates CART mRNA transcription, since the leptin receptor signals through the Janus kinases (JAKs)/STAT pathway (reviewed by Good 2000). The presence of the SP1 adjacent to the STAT-response element (Fig. 1b) in the CART gene promoter sequence adds additional support for this, since SP1 has been shown to play a role in mediating the STAT response (Look *et al.* 1995). Interestingly, a model of leptin action in a thyrotropin-releasing hormone (TRH) neuron includes direct regulation of the TRH promoter by leptin via the phosphorylation of STAT3 (Harris *et al.* 2001). Recently, Elias *et al.* (2001) showed that leptin directly acts on hypothalamic CART neurons that coexpress TRH mRNA.

The Pit-1 binding site at position −818 (Fig. 1a) in the mouse CART promoter suggests the involvement of Pit-1 in cell-type specific transcriptional activation of the CART gene. Pit-1 is a POU-homeobox transcription factor that is responsible for either the commitment or maintenance of somatotroph, lactotroph, and thyrotroph cell lineages (Lin *et al.* 1994; Rhodes *et al.* 1996; Andersen *et al.* 1997). This transcription factor has been shown to be required for the tissue specific expression of several genes including growth hormone, prolactin, and thyrotropin (Haugen *et al.* 1996; Karin *et al.* 1990; Voss and Rosenfeld 1992). The Pit-1 protein has also been shown to participate in synergistic interactions with other transcription factors, including cJun (Farrow *et al.* 1996), thyroid hormone receptor (Chang *et al.* 1996), and estrogen receptor (Ying and Lin 2000). The 1.8-fold increase in luciferase activity exhibited by −3451CART-LUC as compared with −641CART-LUC in GH3 cells (Fig. 2) suggests that Pit-1 may play a role in CART gene expression. In AtT20 cells, −3451CART-LUC resulted in no significant increase in luciferase activity in the presence or absence of forskolin (data not shown). In AtT20 cells, prolactin mRNA is not induced by the Pit-1 pathway (Girardin *et al.* 1998). Additionally, the luciferase activity of −641CART-LUC in GH3 cells (somatomammotroph phenotype) is higher than in AtT20 cells (corticotroph phenotype) thus suggesting that −641CART-LUC contains *cis*-elements that enhance expression in GH 3 cells.

The data obtained from these studies provide a basis for future studies on the mechanisms regulating the CART gene. The data presented (Figs 3 and 4) also confirm that the CART gene can be positively regulated via a cyclic AMP-dependent pathway. Further studies aimed at identifying which transcription factors and pathways are involved in regulating the CART gene may identify common signal transduction pathways that are shared by neuropeptide genes

such as proopiomelanocortin (POMC) and melanin-concentrating hormone (MCH). In the arcuate nucleus CART, POMC, and MCH are coexpressed, affect feeding (Elias et al. 1998; Broberger 1999; Vrang et al. 1999) and may also be transcriptionally regulated in a similar manner.

Acknowledgements

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A patented phytonutrient for weight management by promoting lean body mass

Forskolin Reduces Food Intake In Obese Mice

A randomized placebo controlled study performed at the C. B. Patel Research Center for Chemistry and Biological Sciences, Mumbai , India , revealed that forskolin treatment reduces food intake in obese mice by about 50 percent.

Obese mice treated with a daily dose of 2 mg of forskolin (in two divided doses) reduced their food intake by almost 50 % by the end of the three-week treatment. The researchers concluded that reduction in food intake is likely to be a significant factor contributing to the weight management and lean body mass enhancing effects of forskolin.

In this study, 48 female Swiss Albino mice, weighing 20 - 25 g on an average, were randomly allocated into two groups (study group and control) and housed in groups of four in polypropylene cages in a room maintained at 22 ° C. The animals were exposed to natural day- night cycles. Mice in both groups were fed a highly palatable diet for 12 weeks consisting of 33 % pelleted mice diet, (Lipton India). The study group additionally received 33 % powdered milk (Nestle), 7 % sucrose, 5 % clarified butter (ghee) and 22 % tap water by weight. This diet produced reliable weight gain over controls.

Both the groups were provided food at the rate of 30 g / day / cage divided into two meals. Food intake was measured daily during the treatment period, while body weight and abdominal flab were recorded every seven days. Animals from both groups were randomly selected to receive forskolin or placebo treatment. Treatment was initiated only after the difference in body weight between special obesity-inducing diet fed and control mice was 10g

Administration of forskolin did not produce any untoward hyperactivity or lethargy in mice, although there was significant decrease in food intake in the obese mice.

During the first two weeks of treatment all the mice were consuming on an average 30 g of food per 24 h (normal or obesity inducing) per group of four animals in a given cage. **From the third week of treatment, there was a reduction in the quantity of food consumed by the obese group treated with forskolin. After the third week, the forskolin treated obese animals were consuming about 15 g of the special obesity inducing food per 24 h.**

The intake of water remained unaltered. The mice in all the treatment groups as well as controls were active and performed normally in daily activities throughout the study period.